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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Yawei Ni, et al.

Serial No.: 10/059,627

Filed: January 29, 2002

For: COMBINATION OF A GROWTH FACTOR AND A PROTEASE ENZYME

Group No.: 1655

Examiner: Michael V. Meller

Mail Stop Amendment
Commissioner for Patents
P. O. Box 1450
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Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

YAWEI NI declares that:

1. I am a co-inventor of, and familiar with, the present U.S. Patent Application Serial No. 10/059,627, filed January 29, 2002, in the name of Yawei Ni, and entitled "Combination of a Growth Factor and a Protease Enzyme." I am also familiar with the Official Action dated August 23, 2005 issued therein.

2. I am over 21 years old. I have worked as a Research Scientist/Fellow specializing in carbohydrate polymer, drug delivery, wound healing, and immunology for the last 10 years at Carrington Laboratories Inc. I graduated from Texas A&M University in 1991 with a Ph D in Microbiology/Immunology and a heavy concentration in courses on cell biology.

3. I personally prepared and conducted the examples described in the current patent application.

4. The current patent application relates to achieving a synergistic effect on proliferation and migration of epithelial cells during wound healing process through a stable combination of a protein growth factor related to epithelial cell function (promoting cell proliferation) and a protease capable of clearing extracellular matrix (so that epithelial cells can migrate). As indicated in the current application, there are several growth factors and proteases with the respective functions, including those used in the specifications (see Table 1) and those known to possess the respective functions but not used in the specification (see Table 2). In the future, more growth factors and proteases of such respective functions may be identified. In light of the examples described in the current patent application, any person of ordinary skill in the art would be able to select a growth factor related to epithelial cell function and a protease related to clearance of extracellular matrix to formulate a desired combination of the two corresponding to the claimed subject matter for achieving a synergistic effect. The combinations that could be formulated are not limited to those including only KGF and plasmin (or plasminogen) that are disclosed in Examples 1 - 5. Indeed, the discovery is not only related to the combination of these two particular agents, but more importantly also the combination of two such agents in the respective functional groups for achieving a synergistic effect.

5. The descriptions and teaching of the current patent application provide sufficient guidance for any person of skill in the art to produce viable and effective combinations of growth factors and protease enzymes that are encompassed by the claimed subject matter without undue experimentation. The amount of experimentation required would be well within the accepted amount in this field.

6. All the growth factors and protease enzymes in Tables 1 and 2 can be readily obtained from Sigma Chemical Co. (St. Louis, MO), Calbiochem (San Diego, CA), R&D Systems (Minneapolis, MI), and Peprotech (Rocky Hill, NJ). Based on the invention disclosed in the current

application, one can simply follow Examples 1-4 to mix any one of the growth factors with any one of the proteases from the lists in Tables 1 and 2.

7. Certain combinations would be ineffective for the present invention, because the protease may destroy the growth factor, therefore yielding no stable and effective combination as demonstrated with plasmin and KGF-2 (FGF-10) shown in Example 7. However, it is possible that other combinations would be stable and effective in producing the synergistic effect as with plasmin and KGF shown in Examples 1-4. The destruction of the growth factor by protease or a lack of it can be readily identified by following the examples for performing electrophoretic analysis and biological function testing for the growth factors after the combination (Examples 1 -5). The conditions for the combination, including concentrations of growth factors and proteases, pH and ionic strength of the buffer, and incubation temperature and length, have also been described in the examples and are described again below.

8. Specifically, one can mix 0.1 – 5 μg of a growth factor with a protease at different concentrations from a few nanograms (ng) to a few micrograms (μg) in 20-50 μl reaction volume. Reaction buffer can commonly be a buffered physiological saline at a pH of 7.0-7.4. A control reaction containing no protease should also be included. The mixture is then incubated at 37 °C for 30 min – 120 min and then mixed with gel sample buffer and subjected to gel electrophoresis based on well known standard protein electrophoresis procedures found in the references cited in the application as well as other sources. In particular, see *Current Protocols of Molecular Biology*, Chapter 10 (Ausubel et al., Eds., John Wiley & Sons, 1994 – 2005), a copy of pertinent pages of the Table of Contents is attached as Exhibit 1; and Ron et al., "Expression of Biologically Active Recombinant Keratinocyte Growth Factor: Structure/Function Analysis of Amino-Terminal Truncation Mutants," *J. Biol. Chem.* 268, 2984-2988 (1993), a copy of which is attached is Exhibit 2. A disappearance of the growth factor protein band on the gel without a concurrent appearance of a smaller fragment indicates the complete degradation of the growth factor and therefore that this particular pair of the growth factor and the protease can not be mixed to achieve a stable combination. On the other hand, if the growth factor protein band remains intact or a smaller fragment appears in place of the original growth factor band after the digestion, the growth factor

will be deemed stable in the presence of the protease and thus a combination of the two is feasible. In Examples 1-4, analytical procedures for identifying a stable pair (KGF and plasmin) and an unstable pair (KGF-2 and plasmin) have been provided. Further examples of this analysis can also be found in Sommer and Rifkin, "Interaction of Heparin With Human Basic Fibroblast Growth Factor: Protection of the Angiogenic Protein from Proteolytic Degradation by a Glycosaminoglycan," *Journal of Cellular Physiology* 138, 215 – 220 (1989), a copy of which is attached as Exhibit 3.

9. The feasibility for combination is then readily confirmed using a biological function assay as all growth factors have a certain function. All growth factors including those in Tables 1 and 2 have a biological assay for testing its function as all of them can be measured based on the biological activity. The information and procedure for such assay can be readily found in suppliers' catalogues (listed above), product data sheets, experimental manual books, and literature cited in the application as well as other sources. In particular, see McKay, I.A., "Types of Growth Factor Activity: Detection and Characterization of New Growth Factor Activities," and Van Doelen et al., "Detection of Polypeptide Growth Factors: Application of Specific Bio-Assays and PCR Technology," *Growth Factors: A Practical Approach* (Oxford, NY, IRL Press at Oxford University Press, 1993), copies of both with pages of the Tables of Contents are attached as Exhibit 4; Mosmann, T., "Rapid colorimetric assay for cellular growth and survival: Application to Proliferation and Cytotoxicity Assays," J. Immunol. Methods 65 (1 – 2) 55 – 63, Dec. 16, 1983, a copy of which is attached as Exhibit 5; Martin, Bernice M., *Tissue Culture Techniques: An Introduction* (Boston, Birkauser, 1994), a copy of the Table of Contents is attached as Exhibit 6; Aaronson et al., "Keratinocyte Growth Factor: A Fibroblast Growth Factor Family Member with Unusual Target Cell Specificity," *Annals of the New York Academy of Sciences* 638, 62-77 (1991), a copy of which is attached as Exhibit 7; Rubin, et al., "Purification and Characterization of a Newly Identified Growth Factor Specific for Epithelial Cells," *Proc. Natl. Acad. Sci. USA* 86: 802-806 (1989), a copy of which is attached as Exhibit 8. As an example of a growth factor supplier's catalogue, the data sheets for the growth factors listed in Tables 1 and 2 of R&D Systems' 2006 Catalog (Minneapolis, MN) are attached as Exhibit 9, which provide, among other things, the reference for the biological activity assay. In most cases, the assay is a cell proliferation test using a

certain type of cells such as epithelial cells, since growth factors are protein factors that stimulate cell division or proliferation. The cell proliferation assay used in Example 5 is a good example. Thus, if a growth factor is degraded in a combination with a protease, its biological function will also be lost when tested by the biological assay. On the other hand, if the growth factor is stable in a combination with a protease, its biological function will be intact, thus confirming that a combination of the two is feasible. All these procedures described above can be readily found in published literature and experimental manual books. Information on growth factors and proteases can also be readily found in published literature and product information sheets. All these are well known to one of ordinary skill in the field and can be carried out without any undue experimentation.

10. Together, such combinations can be achieved without any undue experimentation and are indeed within the scope of the discovery as it is understood by those of skill in the art. Again, the invention was made with one pair of growth factor and protease, but the essence of the invention is a combination of a growth factor related to epithelial cell function and a protease related to clearance of extracellular matrix for achieving a synergistic function.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Yawei Ni

02/10/2006

Date

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10.15 Purification of Recombinant Proteins and Study of Protein Interaction by Epitope Tagging

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Expression of Biologically Active Recombinant Keratinocyte Growth Factor

STRUCTURE/FUNCTION ANALYSIS OF AMINO-TERMINAL TRUNCATION MUTANTS*

(Received for publication, August 31, 1992)

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Keratinocyte growth factor (KGF) is a newly identified member of the fibroblast growth factor (FGF) family (FGF-7). KGF is expressed by stromal fibroblasts and acts on epithelial cells in a paracrine mode. To facilitate structure/function studies, we utilized the T7 prokaryotic expression system to synthesize this growth factor. Recombinant KGF (rKGF) was mitogenic with a specific activity around 10-fold higher than native KGF. By *in vitro* mutagenesis, we generated a series of KGF mutants with sequential deletions of the amino-terminal domain, the most divergent region among different FGF members. Mutant proteins, produced in bacteria, were tested for their ability to bind heparin, bind and activate the KGF receptor, and induce DNA synthesis. Heparin binding properties were preserved with deletion of up to 28 amino-terminal residues of the mature KGF but lost by the deletion of an additional 10 residues. Biological activity of mutants with deletions of up to 10 residues was comparable to that of rKGF. However, deletion of 29 residues resulted in significantly reduced ability to stimulate KGF receptor tyrosine-kinase activity and DNA synthesis, although this mutant bound the receptor at high affinity. These characteristics of a partial agonist may be useful in the development of competitive antagonists of KGF action.

Keratinocyte growth factor (KGF)[1] is a recently identified member of the FGF family (FGF-7) (1). Unlike other members of this family with a whole spectrum of target cells, KGF appears to be restricted in its activity to epithelial cells (2). Its expression by stromal fibroblasts of many epithelial tissues suggests a role for KGF as a major paracrine mediator of epithelial cell proliferation. Recently, a high affinity receptor for KGF was cloned and found to be an alternatively spliced isoform of fibroblast growth factor receptor-2 (*bek*/FGFR2) (3, 29). KGF was initially purified from conditioned medium of human embryonic fibroblasts (2). To obtain large quantities of the growth factor required for structure/function studies, we sought to express recombinant KGF in bacteria. We report here a method that produces high yields of functional human recombinant KGF. We have utilized this expression system as a means to generate and characterize KGF mutants with the goal of localizing domains important for KGF biological function.

MATERIALS AND METHODS

Construction of Recombinant Plasmids—Plasmid pet8C (4) was initially used to clone the KGF coding sequence. This insert was adapted for cloning into the *Nco*I/*Bam*HI sites of pet8C by means of the polymerase chain reaction (PCR) (5, 6), as described under "Results." The recombinant plasmid, propagated in HB101 cells, was used to transform BL21(DE3) plys E (4) for induction of KGF expression.

Amino-terminally truncated KGF mutants were generated by the PCR technique (5, 6). A pair of oligonucleotide primers was used to generate each of the mutants; one was derived from the carboxyl-terminal region of the KGF open reading frame common to each mutant: ttg gat cca tta agt tat tgc cat agg aag. The second primer was derived in each case from sequence immediately distal to the region to be deleted (Table I). *Nde*I and *Bam*HI sites were included in the amino-terminal and carboxyl-terminal oligonucleotide primers, respectively, to allow cloning of the amplified inserts into the pet3C plasmid. An *Nde*I site in the KGF coding sequence was first eliminated using two steps of site-directed mutagenesis which did not change the primary amino acid sequence.

DNA was amplified by PCR as described previously (6). Briefly, 1 ng of plasmid DNA containing KGF cDNA was used as a template. Amplification was performed for 25 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 3 min in a total volume of 100 μl. The amplified DNA was purified by Sephadex G-50 spin columns and subjected to restriction enzyme digestion to allow cloning into the appropriate vectors. Plasmid DNA was prepared from positive colonies, and the nucleotide sequence of each mutant was confirmed using the dideoxy sequencing method (7).

Production and Purification of Recombinant Human KGF—

TABLE I

Second primer generated by PCR

The second primer was derived from the sequence immediately distal to the region to be deleted.

Mutant	Deletion	Primer
K10	1-34	atc ata tga ctc cag agc aaa tg
K11	1-39	atc ata tgg aag gag ggg ata ta
K12	1-58	atc ata tgg aag gag ggg ata ta
K13	1-69	atc ata tgt ttt gtc gaa cac agt ggt ac
K14	1-79	atc ata tga aaa gag gca aag ta

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[1] The abbreviations used are: KGF, keratinocyte growth factor; rKGF, recombinant KGF; FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

BL21(DE3) plys E cells bearing recombinant plasmid were grown at 37 °C in terrific broth (8) containing 100 μg/ml ampicillin. When A_{595} reached 1.0, isopropyl 1-thio-β;-D-galactopyranoside (1 mM) was added, cultures were incubated for 3 h, and cells were collected by centrifugation. The cell pellet was resuspended in TENG buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.5) containing protease inhibitors, and the cells were lysed by three successive cycles of freezing and thawing. The lysate was disrupted by sonication, cleared by centrifugation, and the supernatant was frozen prior to growth factor purification.

Purification of recombinant KGF was performed by applying the bacterial supernatant to a heparin-Sepharose column (4-ml bed volume). The column was washed with phosphate buffer (50 mM, pH 7.2, containing 0.2 M NaCl) until the absorbance fell to near base line and then subjected to a linear step gradient of increasing NaCl concentration. Aliquots from fractions were analyzed by SDS-PAGE and tested for mitogenic activity. Selected fractions were concentrated 10–20-fold with a Centricon-10 microconcentrator (Amicon). The concentrated fractions were diluted in 50 mM phosphate buffer, pH 6.8, and subjected to fast protein liquid chromatography/Mono S cation exchange chromatography using a gradient of increasing NaCl concentration. rKGF was eluted around 0.45 M NaCl. The amino acid composition and final concentrations of purified KGF and KGF mutants were determined by amino acid analysis.

Protein Detection, Binding Assays, and Mitogenic Activity—Polyacrylamide gels were prepared with SDS by the procedure of Laemmli (9). Samples were boiled for 3 min in the presence of 2.5% (v/v) 2-mercaptoethanol. The gels were fixed and silver stained (10) by using the reagents and protocol available from Bio-Rad, or they were transferred without fixation to polyvinylidene difluoride membranes for immunoblotting. Rabbit polyclonal antisera raised against full-length KGF or synthetic peptides corresponding to KGF residues 33–44 or 179–194 (where residue 1 is the initiation codon in the KGF open reading frame) were used for KGF detection. Immunoprecipitation and immunoblotting of cellular proteins phosphorylated on tyrosine in response to KGF were performed as described previously (11).

^{125}I-KGF binding and competition experiments were performed using NIH/3T3 cells transfected with the murine KGF receptor as described previously (3, 11). DNA synthesis was measured by [^{3}H] thymidine incorporation. Balb/MK cells, plated in 96-well microtiter plates, were serum starved for 48 h, treated with growth factor, and processed as described previously (2).

RESULTS

Construction of a Prokaryotic KGF Expression Vector—For prokaryotic expression of KGF, we utilized the pet8c vector containing the promoter of the highly expressed φ10 gene of bacteriophage T7 (4). This vector contains the first 11 amino acids of φ10 as well as a Tφ transcription termination signal recognized by the T7 RNA polymerase. The amino-terminal residues of the T7 φ10 gene product were excised, and one of the upstream cloning sites was used to express the KGF gene product. The sequence corresponding to KGF residues Ala^{31} to Thr^{194} was amplified by PCR. The 5′ and 3′ ends of the DNA were designed to include *Nco*I and *Bam*HI recognition sites, respectively, to allow cloning of the insert into the *Nco*I/*Bam*HI sites of pet8c. An amino-terminal methionine codon was included for initiation of translation. This construct was designated k4–9 (Fig. 1).

The amino acid sequence of mature KGF purified from human fibroblast culture fluids consists of Cys^{32}–Thr^{194}, numbered from the KGF initiation codon (1). We included Ala^{31} in our prokaryotic expression vector because the codon which follows the AUG can influence the translation efficiency of prokaryotic proteins (12). Cys in this position has been shown to reduce translation efficiency, and it rarely exists in this position in highly expressed proteins (12). In contrast, Phe, Ala, and Lys are commonly found in this position, and these amino acids do not decrease translation efficiency.

Expression and Characterization of Recombinant KGF—We initially attempted to express recombinant KGF (rKGF) in

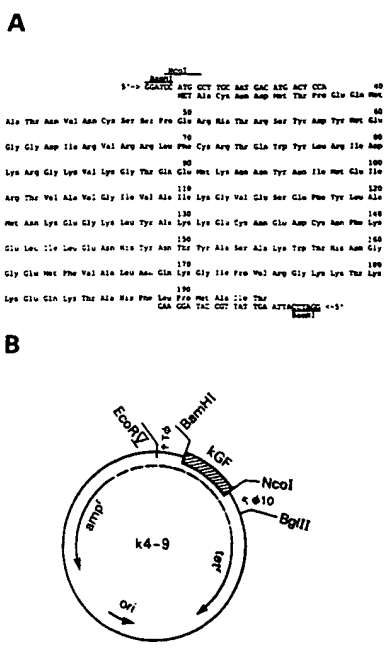

FIG. 1. **Construction of the k4–9 KGF expression vector.** *Panel A*, the amino acid sequence of recombinant KGF. Primers used for amplification are indicated at the amino and carboxyl termini of the *inset*. The numbering corresponds to that of the native KGF polypeptide. *Panel B*, schematic representation of the KGF expression vector. The KGF coding sequence was cloned into the *Nco*I and *Bam*HI sites of the pet8c vector, which contains the promoter of the T7 φ10 gene and the φ10 transcription termination signal. *amp^r*, ampicillin resistance gene; *tet^r*, tetracyclin resistance gene; *ori*, origin of replication.

BL21(DE3) plys E cells. However, KGF was highly toxic to these cells, and their growth was dramatically inhibited even by basal levels of KGF expression in the absence of isopropyl 1-thio-β;-D-galactopyranoside induction. We next analyzed BL21(DE3) plys E cells, which express a T7 RNA polymerase-specific inhibitor, the T7 lysozyme (13). This inhibitor reduces the basal level of target gene expression prior to induction and thereby minimizes potential toxic effects (4). Since expression of the T7 lysozyme is also driven by the T7 RNA polymerase promoter, its level of expression also increases upon induction, resulting in slower accumulation of the target protein (4). In this system, by 4 h postinduction, rKGF comprised 1–2% of the total cellular protein (data not shown).

rKGF was purified as described under "Materials and Methods" and eluted from heparin-Sepharose in 0.5 M NaCl as determined by mitogenic assay on Balb/MK cells. The elution profile from the Mono S cation exchange column revealed two protein peaks, both of which had mitogenic activity on Balb/MK cells (Fig. 2*A*). Silver staining of the Mono S peak fractions showed that rKGF was purified to homogeneity (Fig. 2*B*, *fourth* through *seventh lanes*). The apparent molecular mass of the polypeptide from the first peak was around 21 kDa, as expected for the intact rKGF product, whereas that of the polypeptide(s) eluted in the second peak was 16–17 kDa (Fig. 2*B*, *eighth* and *ninth lanes*).

Amino acid analysis of the 21-kDa protein observed by silver stain revealed that its composition was in good agreement with the predicted KGF sequence (data not shown). To prove that the 21-kDa protein was indeed rKGF and to investigate the smaller protein(s) further, we subjected the Mono S peak fractions to immunoblot analysis. As shown in Fig. 3*A*, antiserum directed against purified KGF readily recognized both the 21-kDa and smaller recombinant protein(s). In contrast, antiserum raised against an amino-ter-

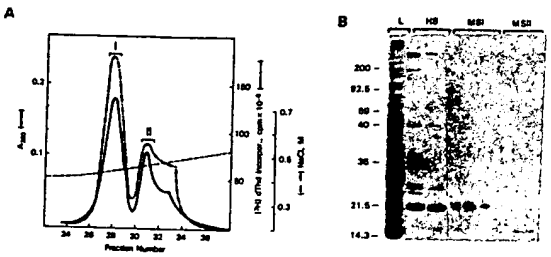

FIG. 2. **Purification of rKGF.** *Panel A*, mitogenically active fractions eluted from the heparin-Sepharose column were processed as described under "Materials and Methods," loaded onto a Mono S ion exchange column, and eluted with a linear gradient of increasing NaCl concentration. The Mono S elution profile is indicated by the *solid line*. Biological activity (*dashed line*) was determined by measuring the incorporation of [^{3}H]thymidine into Balb/MK cells. Two distinct peaks of biologically active protein were obtained (indicated by numerals *I* and *II* above the elution profile). *Panel B*, 14% SDS-PAGE analysis of selected fractions from the heparin-Sepharose and Mono S columns. Proteins were detected by silver stain as described under "Materials and Methods." *L*, bacterial lysate; *HS*, peak fractions from heparin-Sepharose column; *MSI*, Mono S peak I (indicated in *panel A*); *MSII*, Mono S peak II (indicated in *panel A*).

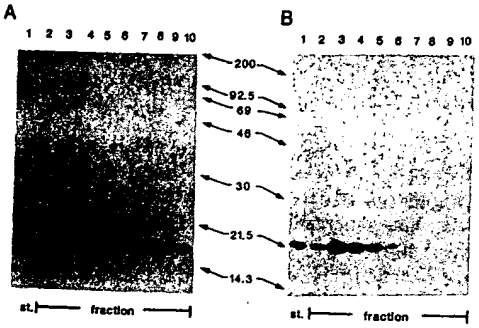

FIG. 3. **Immunodetection of rKGF.** Starting material (*st.*) and peak fractions from the Mono S column were analyzed by SDS-PAGE, transferred to nitrocellulose, and rKGF was detected using KGF antiserum raised against the entire molecule (*panel A*) or against an amino-terminal synthetic peptide corresponding to KGF residues 33–44 (*panel B*).

TABLE II

Response of Balb/MK cells to mitogens in the presence or absence of heparin

Data are from one representative experiment performed in triplicate, and are expressed as mean cpm ± S.D.

Treatment	[^{3}H]Thymidine incorporation	
	−Heparin	+Heparin[a]
	cpm	
Background	170 ± 64	136 ± 8
rKGF (5 ng/ml)	76,841 ± 6,254	17,829 ± 4,140
M426 KGF[b]	73,659 ± 8,330	13,090 ± 4,507
EGF (10 ng/ml)	20,004 ± 2,665	13,135 ± 765
aFGF (10 ng/ml)	20,550 ± 4,128	47,645 ± 13,217

[a] Concentration of heparin = 20 μg/ml (~3 units/ml).
[b] Estimated to be approximately an order of magnitude higher concentration than bacterially expressed KGF.

minal KGF peptide recognized only the 21-kDa protein (Fig. 3*B*). Taken together, these results established that both recombinant proteins were KGF products and that the smaller proteins reflected proteolysis of the KGF amino terminus.

A hallmark of FGF family members is their ability to bind heparin. For aFGF and *hst*, it has been reported that heparin can augment their biological activities (14, 15). Thus, we examined the effect of heparin on the mitogenic activity of native and recombinant KGF. Table II shows that, unlike aFGF and *hst*, the mitogenic activities of both naturally occurring and rKGF were significantly inhibited by heparin. When the concentration dependence of rKGF mitogenic activity on Balb/MK cells was compared with that of the native protein purified from M426 fibroblasts, we found that the dose required for half-maximal stimulation by rKGF was 1 ng/ml. This was in striking contrast to native KGF, which required a 10-fold higher concentration to achieve a similar level of activity (Fig. 4*A*). The molecular masses observed for rKGF and native KGF were 21 and 26–28 kDa, respectively (Fig. 4*B*). The faster mobility of rKGF likely reflects the absence of glycosylation of the bacterially expressed protein. Whether this accounts for its greater specific mitogenic activity remains to be determined.

Expression and Characterization of KGF Amino-terminal Mutants—Comparison of the predicted amino acid sequence of KGF with that of other FGF family members revealed greatest divergence from KGF in their amino-terminal domains. Since amino-terminal proteolytic digestion resulted in rKGF products with mitogenic activity, we sought to study more thoroughly the effects of sequential amino-terminal deletions on KGF biological properties. Mutants were generated by PCR as described under "Materials and Methods." We took advantage of the presence of Met codons within the KGF open reading frame at positions 35, 40, and 59 and deleted sequences 5′ of each of these codons. Two additional mutants were engineered in which the first 70 or 80 residues of the mature KGF polypeptide were deleted, and an AUG codon was added to initiate translation. A schematic diagram of each mutant is shown in Fig. 5*A*.

K10, K11, and K12 retained the ability to bind heparin and eluted at the same salt concentration as the parental molecule (data not shown). Whereas K13 and K14 mutants were expressed at levels similar to wild type rKGF (Fig. 5*B*), these mutants completely lost the ability to bind heparin. Attempts to purify K13 and K14 using Mono S or CM-Sephadex resins were also unsuccessful. Since K13 and K14 lacked detectable mitogenic activity, they were not characterized further. K10, K11, and K12 mutants were purified to homogeneity by Mono S chromatography (Fig. 5*C*) and their identities confirmed by immunoblot analysis using rabbit polyclonal antisera directed against a synthetic peptide derived from the KGF carboxyl-terminal sequence (Fig. 5*D*).

We next investigated the abilities of K10, K11, and K12 mutants to displace radiolabeled KGF from its receptor, trigger receptor-kinase activity, and stimulate Balb/MK DNA synthesis. Each of the mutants effectively displaced ^{125}I-KGF bound to NIH/3T3 cells overexpressing the KGF receptor

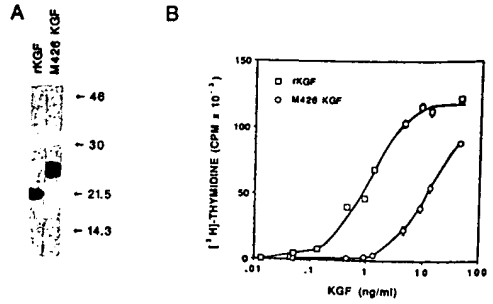

FIG. 4. **Comparison of the molecular weights and mitogenic activities of recombinant and native KGF.** *Panel A*, following SDS-PAGE, recombinant (*left lane*) and native (*right lane*) proteins were transferred to nitrocellulose and immunoblotted with antisera raised against native KGF purified from M426-conditioned media. *B*, mitogenic activity was assessed using Balb/MK cells as described under "Materials and Methods."

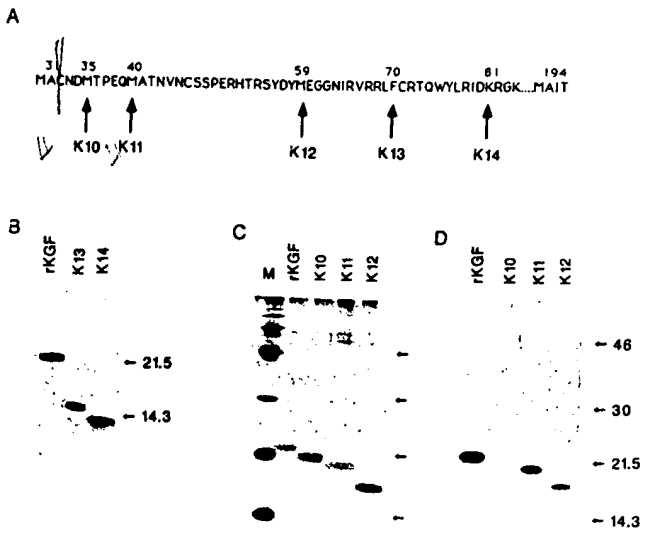

FIG. 5. **Generation and expression of amino-terminal truncation mutants of KGF.** *Panel A, arrows* indicate the sites of amino-terminal truncation for KGF mutants K10–K14. The *numbers* above the sequence refer to KGF residues as predicted from the M426 cDNA coding sequence. For rKGF, K13, and K14, an ATG codon was added to initiate translation (see "Materials and Methods"). *Panel B*, anti-KGF immunoblot analysis of the level of expression of K13, K14, and rKGF in bacterial lysates. Molecular mass markers are indicated in kDa at *right*. *Panel C*, silver-stained SDS-PAGE gel of purified recombinant proteins. *M*, molecular mass standards. *Panel D*, immunodetection of recombinant proteins with an antipeptide sera directed against the carboxyl terminus of KGF. Molecular mass markers for *panels C* and *D* are indicated in kDa by *arrows* at *right*.

(Fig. 6*A*). We have reported previously that KGF induces tyrosine phosphorylation of a 90-kDa protein (11). As shown in Fig. 6*B*, mutant K10 induced tyrosine phosphorylation of pp90 to the same extent as rKGF. Similar results were obtained with mutant K11 (data not shown). However, phosphorylation of pp90 by K12 was significantly less than observed with rKGF or K10 under the same conditions (Fig. 6*B*).

As shown in Fig. 6*C*, each of the mutant proteins induced DNA synthesis in quiescent Balb/MK cells. However, only mutants K10 and K11 were as potent as rKGF. The half-maximal activities of rKGF, K10, and K11 were in the range of 1–2 ng/ml. In contrast, K12 displayed 10–20-fold reduced mitogenic activity. To exclude the possibility that the lower biological activity of mutant K12 was because of its instability, we incubated equal amounts of KGF or K12 at 37 °C in Dulbecco's modified Eagle's medium in the presence or absence of Balb/MK cells for 48 h. The medium was then removed, clarified by centrifugation, and tested for mitogenic activity. We observed no evidence that K12 was relatively more labile by this type of analysis.

DISCUSSION

We demonstrate that recombinant expression of KGF in bacteria results in efficient production of biologically active growth factor. We estimate that in the inducible system utilized, rKGF represented as much as 1–2% of bacterial proteins. By means of heparin-Sepharose chromatography, it was possible to achieve a preparation that was approximately 90% pure. Subsequent Mono S cation exchange chromatography led to an essentially homogeneous rKGF preparation, making this system practical for producing large quantities of pure recombinant growth factor.

Recombinant KGF was substantially more active as a mitogen than the native molecule. Since both proteins were purified to homogeneity, the 10-fold greater specific activity of rKGF likely reflects differences in intrinsic properties. Consistent with the presence of a potential Asn-linked glycosylation site in its predicted amino acid sequence (1), KGF expressed in mammalian cells is glycosylated, as evidenced by its increased electrophoretic mobility when synthesized by cells exposed to tunicamycin.[2] Thus, the smaller apparent size of rKGF as compared with the native molecule likely reflects lack of glycosylation in the bacterial expression system. Whether the absence of glycosylation is responsible for the greater specific mitogenic activity of rKGF remains to be elucidated.

The mitogenic activities of both rKGF and native KGF were found to be strongly inhibited by heparin. Yet, heparin has been shown to protect aFGF and bFGF from heat inactivation and proteolytic digestion (for review see Ref. 14) and to stabilize K-FGF/HST/FGF-4 (15). Recently, it was reported that interaction with heparin or heparin sulfate type proteoglycans is essential for bFGF binding to its high affinity receptor as well (16, 17). However, the effect of heparin on bFGF mitogenic activity appears to be cell type-dependent. Shipley *et al.* (18) showed that heparin concentrations as high as 10 μg/ml had no effect on bFGF mitogenic activity for human fibroblasts but markedly reduced its activity on human

[2] D. Ron, unpublished data.

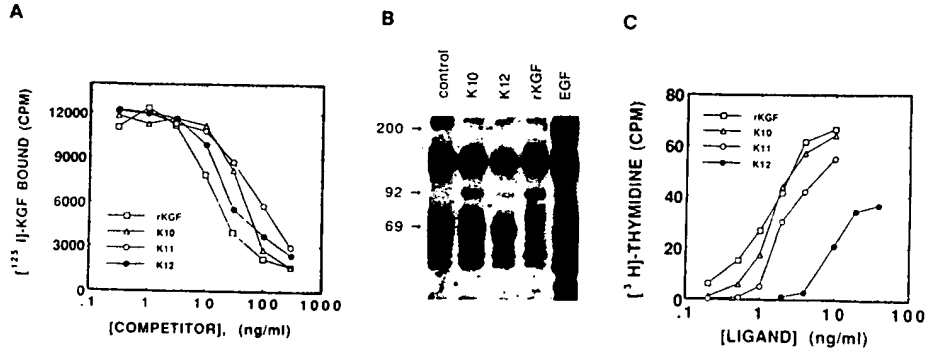

FIG. 6. **Biological activities of KGF amino-terminal truncation mutants.** *Panel A*, competition for ^{125}I-rKGF binding to NIH/3T3 cells overexpressing KGF receptor by unlabeled rKGF, K10, K11, and K12. *Panel B*, autoradiogram of phosphotyrosyl proteins from intact Balb/MK cells following treatment with K10, K12, and rKGF for 10 min at 37 °C and untreated control cells. Cells were lysed, immunoprecipitated with anti-phosphotyrosine, and processed for SDS-PAGE and immunoblotting as described under "Materials and Methods." *Panel C*, mitogenic activity of rKGF, K10, K11, and K12 on Balb/MK cells as a function of ligand concentration. [^{3}H]Thymidine incorporation assays were performed as described under "Materials and Methods."

keratinocytes (18). Heparin inhibition of bFGF mitogenic activity has been observed with Balb/MK cells as well (19). Thus, it is possible that the inhibitory effect of heparin on KGF mitogenic activity also may be cell type-dependent.

By analysis of a series of genetically engineered or naturally occurring rKGF amino-terminal truncation mutants, it was possible to define the extent to which such residues contribute to KGF heparin binding properties and mitogenic activity. Deletion of as many as 28 residues from the amino terminus of the mature molecule did not affect heparin binding properties, but deletion of an additional 10 amino acids resulted in complete loss of heparin binding. This domain contains 3 positively charged amino acids, which might contribute directly to heparin binding. Efforts to localize heparin binding sites in aFGF and bFGF have led to evidence that their carboxyl-terminal halves are involved (20–24). Since these molecules share significant similarity to KGF, it is possible that the amino-terminal deletion resulting in loss of KGF heparin binding affects the tertiary structure in such a way as to affect heparin binding sites indirectly in the carboxyl terminus of the molecule. Thus, more subtle mutagenesis will be required to localize KGF heparin binding domains precisely.

Mitogenic activity was retained by K10 and K11 mutants as well as by amino-terminal proteolytic digestion products of rKGF, whose mobilities suggest loss of the first 18–19 amino-terminal residues. The K12 mutant, which lacked 28 residues, retained the capacity for high affinity receptor interaction but showed substantially decreased ability to activate the receptor kinase and stimulate cell DNA synthesis. A previously reported aFGF mutant with a Gly substitution for Lys^{132} (22) has been reported to exhibit substantially reduced mitogenic activity but no detectable loss of ability to stimulate early events including tyrosine phosphorylation of p90 and phospholipase Cγ as well as the transcriptional activation of *fos* and *myc* genes (22, 25). Thus, both mutants imply that high affinity receptor binding is not sufficient to initiate the cascade of biochemical events required for a mitogenic response.

Accumulating evidence indicates that a number of growth factors activate their receptors by inducing receptor dimer formation (for review see Ref. 26). This appears to be the case for FGF receptors as well (30). The most well studied ligand receptor interactions involve growth hormone and its receptor, in which crystallography of the ligand-receptor complex has revealed that two separate sites in the growth hormone molecule bind to identical sites on two receptors (27). Recently, a potent competitive antagonist of growth hormone action has been generated by mutagenesis which enhances site 1 and diminishes site 2 binding. By this approach, the antagonist competes effectively for receptor binding and blocks dimer formation required for receptor activation (28). It will be of interest to determine whether the K12 mutant acts as a partial agonist through a similar mechanism. The ability to generate large quantities of rKGF should make it possible to explore KGF structure/function relationships further as well as to investigate possible applications of this growth factor to clinical conditions requiring enhanced epithelial cell renewal.

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Interaction of Heparin With Human Basic Fibroblast Growth Factor: Protection of the Angiogenic Protein From Proteolytic Degradation by a Glycosaminoglycan

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Fibroblast growth factors (FGF) are a family of heparin-binding angiogenic polypeptide mitogens. In the presence of heparin, recombinant human basic fibroblast growth factor (bFGF) is fully protected from tryptic digestion and partially protected from chymotryptic digestion. Complete protection of bFGF by heparin is achieved at ratios of growth factor:heparin of approximately 10 or less (w/w). The protection requires bioactive bFGF because inactivated bFGF is rapidly degraded by trypsin or chymotrypsin in the presence of heparin. The bFGF-heparin interaction forms hydrophobic complexes which become insoluble in aqueous buffers at bFGF:heparin ratios of 8 to 10 (w/w). The heparin was found to bind up to a tenfold excess of bFGF on a weight basis. bFGF in the presence of heparin is as active as bFGF alone in inducing ^{3}H-thymidine incorporation into Swiss 3T3 fibroblast DNA.

Fibroblast growth factors (FGF) are angiogenic polypeptide mitogens with a broad target-cell specificity (for reviews see Folkman and Klagsbrun, 1987; Gospodarowicz et al., 1987; Baird et al., 1986). FGFs bind strongly to heparin (Maciag et al., 1984; Shing et al., 1984), and this property facilitates their purification from many tissues and cell lines (Lobb et al., 1986). Based in part on the specific affinity for heparin, FGFs can be classified either as acidic (aFGF) or basic fibroblast growth factors (bFGF) (Klagsbrun and Shing, 1985). Purified FGFs are structurally and functionally labile proteins: they are readily cleaved by proteases (Klagsbrun et al., 1987; Sommer et al., 1987) and are rapidly inactivated by heat and low pH (Gospodarowicz and Cheng, 1986). At 37°C and at neutral pH, the half-life in vitro of FGF is reported to be 24 hours (Westall et al., 1983).

The interaction of heparin with aFGF has been shown to potentiate its activity (Schreiber et al., 1985; Thornton et al., 1983) and restores bioactivity to inactive growth factor. Heparin also protects both aFGF and bFGF from heat- and acid-inactivation (Gospodarowicz and Cheng, 1986). Human bFGF bound to endothelial cell-derived heparan sulfate is protected from degradation by plasmin (Saksela et al., 1988). Although the physiological significance of the interaction of FGF with heparin and/or heparan sulfate is not clear, it is possible that in vivo FGF molecules are protected by binding to glycosaminoglycans. FGF has indeed been identified in the subendothelial extracellular matrix (Baird and Ling, 1987; Vlodavsky et al., 1987).

This report describes the interaction of recombinant human bFGF with heparin with respect to a) proteolytic degradation of bFGF by trypsin and chymotrypsin; b) binding saturation of heparin with bFGF; c) solubility of heparin-bFGF complexes; and 3) the mitogenic activity of bFGF in the presence of heparin.

MATERIALS AND METHODS

Recombinant human bFGF was prepared as reported previously (Squires et al., 1988) and stored in lyophilized form at −70°C. TPCK-treated trypsin and alpha-chymotrypsin were purchased from Sigma (St. Louis, MO). Heparin from porcine intestinal mucosa was obtained from Sigma (177 USP units/mg) and Elkins (Sinn, Inc., Cherry Hill, NJ, 10,000 UPS units/ml).

Formation of FGF-Heparin Complexes

Lyophilized recombinant human bFGF was reconstituted in 50 mM Tris-Hcl, pH 7.5, to a final protein concentration of 2 mg/ml. Heparin was resuspended in distilled water to a concentration of 10 mg/ml. This stock solution or dilutions thereof was stored frozen at −20°C. Heparin was added to bFGF protein solutions to give protein:heparin ratios as indicated in the results section. For some experiments the bFGF was thermally inactivated prior to the addition of heparin. Thermal inactivation of the bFGF has been accomplished by exposure of the growth factor to tempera-

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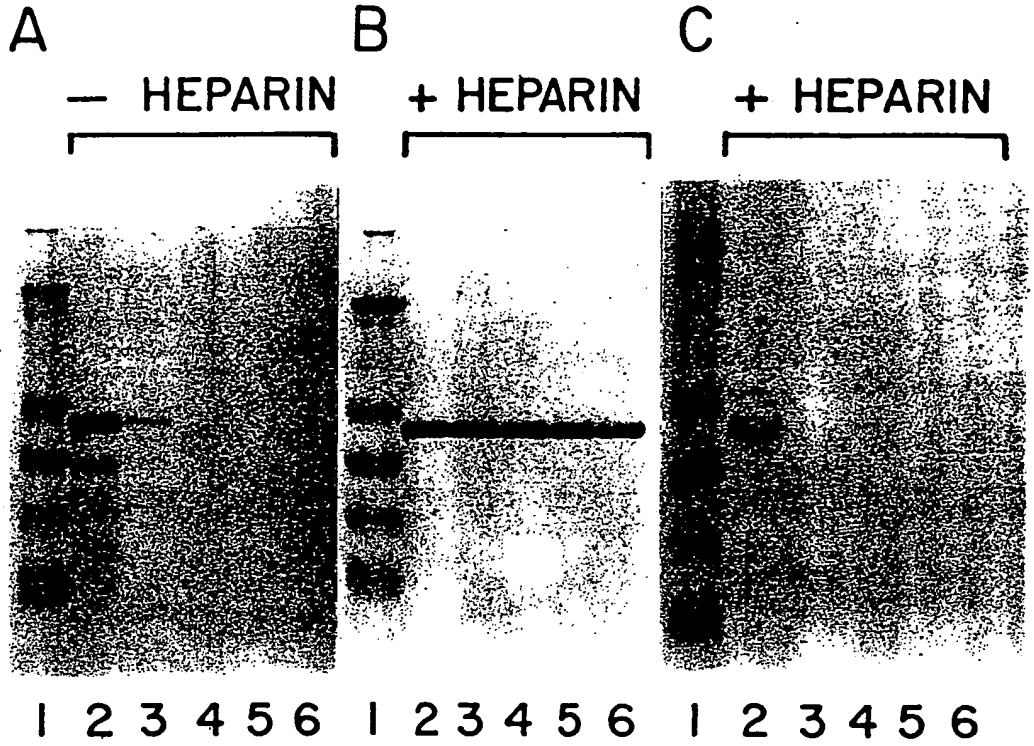

Fig. 1. Heparin protection of bFGF from degradation by trypsin. Bioactive bFGF (A,B) or inactivated bFGF (C) were exposed to 2% (wt/wt) trypsin in the absence (A) or presence (B,C) of heparin (bFGF:heparin ratio = 5). Trypsin digestion was carried out at 37°C and aliquots of each digestion mixture were analyzed by SDS-PAGE after 1-min (lanes 2), 30-min (lanes 3), 180-min (lanes 4), 300-min (lanes 5) and 480-min (lanes 6) exposure to trypsin.

tures of 37°C for 4 days or 65°C for 1 min. Either treatment results in a) loss of bioactivity (mitogenicity assay) and b) loss of heparin-binding ability of the growth factor (A. Sommer, unpublished data).

Proteolytic Digestion of bFGF or bFGF-Heparin Complexes

Solutions of bFGF alone or in the presence of heparin were equilibrated at 37°C for 5 min. Trypsin or chymotrypsin was added to a final concentration of 2 μg protease/100 μg bFGF and digestion was allowed to proceed at 37°C. At various times (see Results) samples were removed from the digestion mixtures and added to an equal volume of SDS gel sample buffer. The samples were immediately heated at 65°C for 15 min and subjected to SDS-polyacrylamide gel electrophoresis.

SDS-PAGE

SDS-polyacrylamide gels (17.5%) (Laemmli, 1970) were stained with Coomassie blue. The quantity of bFGF in a given lane was estimated with an LKB laser scanner.

Mitogenicity Assay

Mitogenicity was measured by the incorporation of ^{3}H-thymidine in the following way: 2.5×10^4 Swiss 3T3 cells in 0.5 ml Dulbecco's modified Eagle's medium (DMEM), 10% calf serum (Colorado Serum Co.) were placed into the wells of 48-well tissue culture plates (Costar). Four days following plating, the medium was aspirated and replaced with fresh DMEM, 0.5% calf serum. Twenty-four hours later, bFGF or bFGF-heparin complexes diluted in PBS containing 0.1 mg/ml gelatin were added in 50-μl volumes. Eighteen to 20 hours following the addition of the bFGF, the culture medium was aspirated and replaced with 500 μl fresh DMEM, 10% calf serum, and 0.5 μCi of ^{3}H-thymidine. After a 2–3-hour labeling period the medium was aspirated, and the cells washed once with 0.5 ml PBS per well. The cells were then treated twice with 0.5 ml of ice-cold 5% trichloroacetic acid (TCA) (10 min each treatment) followed by one 0.5-ml wash with distilled water. The cells were solubilized with 0.5 ml of 0.3N NaOH (1 hour at room temperature) and 0.4 ml from each well was counted in a Beckman scintillation counter.

RESULTS

The degradation of recombinant human bFGF by trypsin was assessed in the absence or presence of heparin (Fig. 1). Bioactive bFGF was rapidly degraded by trypsin (Fig. 1A, lanes 2–6) but was protected from cleavage by the protease in the presence of heparin (bFGF:heparin ratio = 5 (w/w)) (Fig. 1B, lanes 2–6). The observed protection depends on bioactive bFGF because thermally inactivated growth factor is rapidly digested in the presence of heparin (Fig. 1C, lanes 2–6). The latter observation rules out the possibility that

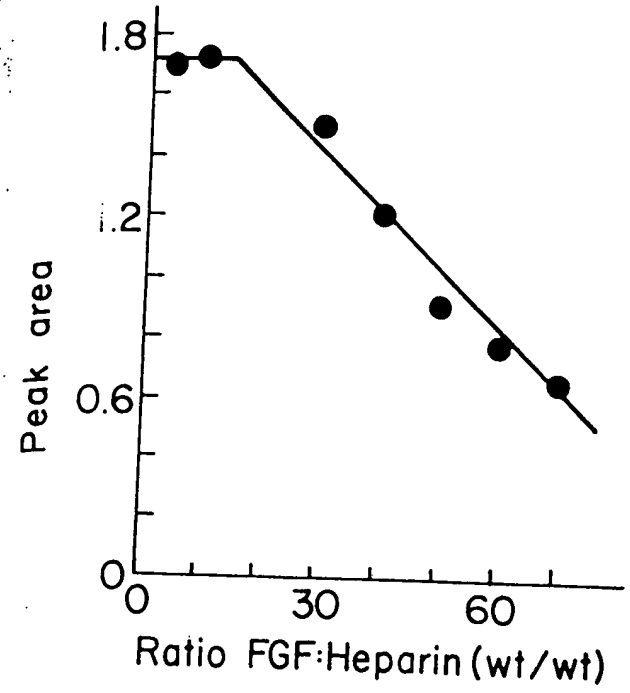

Fig. . Minimum heparin requirement for protection of bFGF from tryptic digestion. Heparin was added to bFGF samples to form bFGF:heparin ratios as shown. Trypsin was added to each sample followed by incubation at 37°C for 4.5 hours. Aliquots of each digestion mixture were subjected to SDS-PAGE. Undigested bFGF in each lane of the gel was estimated by laser scanning and plotted as a function of the protein:heparin ratio of the corresponding digestion mixture.

heparin inactivates the trypsin. To estimate the minimum amount of heparin required to protect bFGF from degradation, a series of samples containing equal amounts of bFGF were supplemented with decreasing amounts of heparin so as to generate a series of bFGF:heparin ratios (w/w) ranging between 5 and 80. Each sample was exposed to 2% trypsin at 37°C for 4.5 hours. Aliquots from each digestion mixture were then analyzed by SDS-polyacrylamide gel electrophoresis. The relative quantity of undigested bFGF in each sample was estimated by laser scanning of the polyacrylamide gel and plotted as a function of the bFGF:heparin ratio of the relevant digestion mixture (Fig. 2). The graph indicates that bFGF is protected from trypsin degradation when present at levels of up to 10–13-fold excess over heparin.

Although initial experiments were carried out with heparin from Sigma, similar results have been obtained using heparin from Elkins (S. Blei, unpublished data). This heparin has been reported to be more active than heparin from Sigma (Castellot et al., 1986).

The protective effect could be achieved by interaction of the negatively charged sulfate and carboxyl groups of the glycosaminoglycan with the positively charged side chains of bFGF lysines and arginines which, in the absence of heparin, are recognized as cleavage sites by trypsin. The susceptibility of bFGF-heparin complexes to chymotrypsin, a protease specific for hydrophobic amino acids such as tryptophane, tyrosine, phenylalanine, leucine, methionine and histidine was also investigated. bFGF-heparin complexes (protein:heparin ratio = 5 (w/w)) were exposed to chymotrypsin in the same manner as described above for trypsin.

The chymotrypsin digestion results are shown in Figure 3. In the absence of heparin, the bioactive bFGF

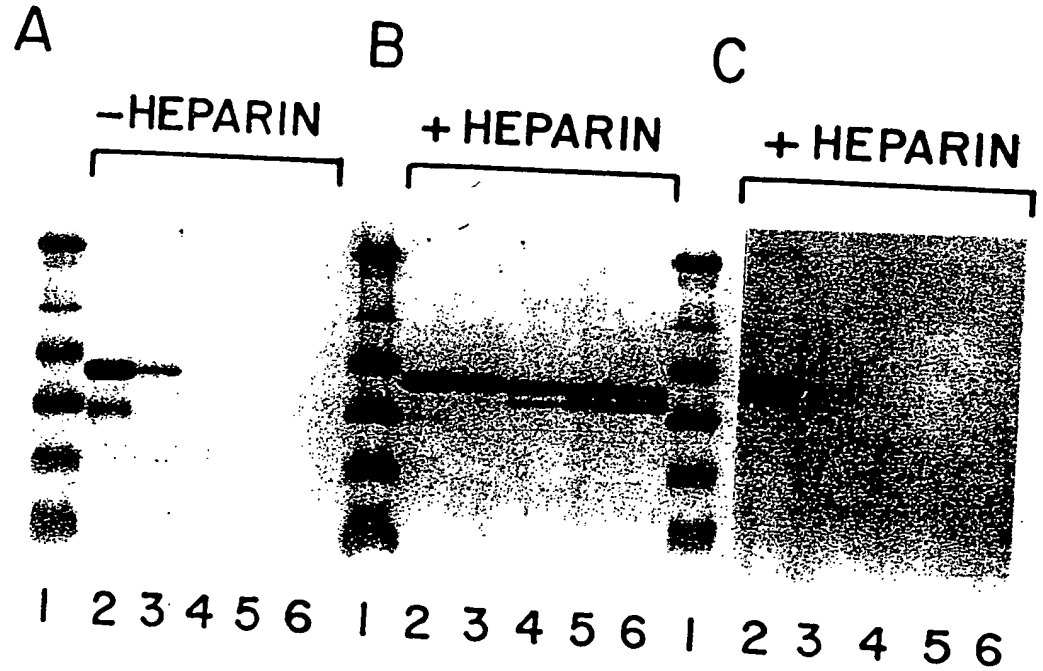

Fig. 3. Heparin protection of bFGF from degradation by chymotrypsin. Bioactive bFGF (A,B) or inactivated bFGF (C) were exposed to 2% chymotrypsin in the absence (A) or presence (B,C) of heparin (bFGF:heparin ratio = 5). Chymotryptic digestion was carried out at 37°C and aliquots of each digestion mixture were analyzed by SDS-PAGE after 1-min (lanes 2), 30-min (lanes 3), 180-min (lanes 4), 300-min (lanes 5), and 480-min (lanes 6) exposure to chymotrypsin.

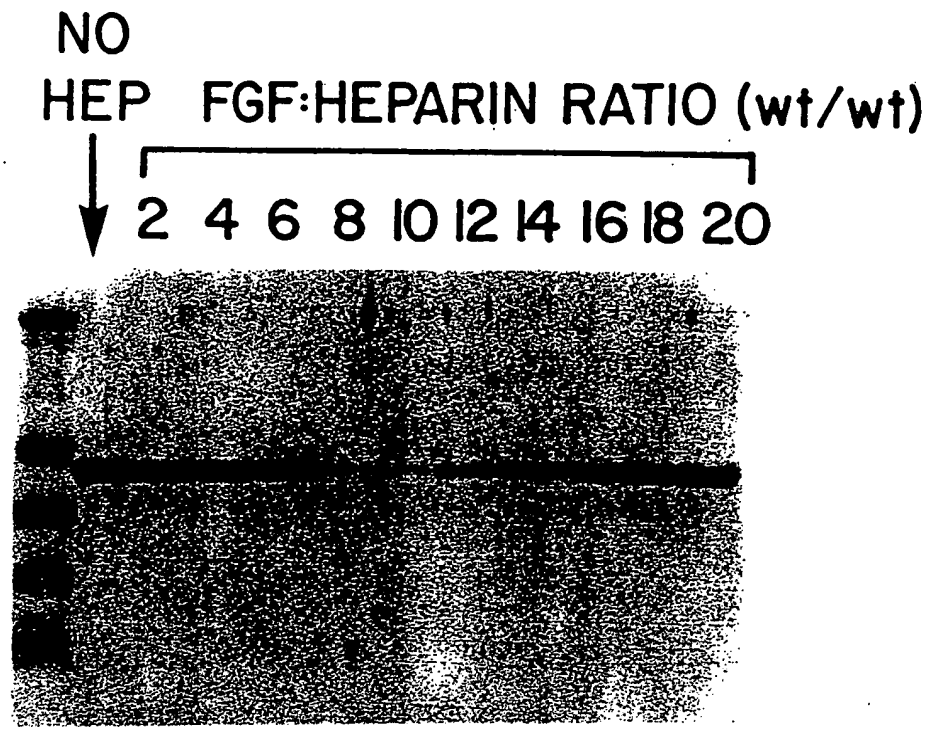

Fig. 4. Solubility of bFGF-heparin complexes. bFGF and heparin were mixed to generate bFGF:heparin ratios in the range of 2 to 20. The mixtures were incubated at room temperature for 30 min. Insoluble complexes were removed by centrifugation, and aliquots of the soluble supernatants were analyzed by SDS-PAGE.

is rapidly degraded by the protease (Fig. 3A, lanes 2–6). In the presence of heparin, the bFGF is only partially protected from degradation (Fig. 3B, lanes 2–6). The partial protection depends on bioactive bFGF because inactivated growth factor is rapidly degraded in the presence of heparin (Fig. 3C, lanes 2–6). Although the bFGF-heparin complexes are susceptible to cleavage by chymotrypsin, the degradation pattern is not random since a truncated form of the bFGF accumulates over the 8 hour digestion period (Fig. 3B, lanes 4–6).

To estimate the bFGF binding capacity and to test the solubility of the protein-heparin complexes, a series of samples was prepared with bFGF:heparin ratios in the range of 2 to 20 (w/w). The mixtures were incubated for 30 min at room temperature and centrifuged for 2 min. Each supernatant was analyzed for the presence of bFGF by SDS-polyacrylamide gel electrophoresis (Fig. 4) The gel shows that bFGF-heparin complexes formed at ratios of 2 to 6 remain soluble. At bFGF: heparin ratios of 8 to 10, most of the complexes formed precipitate and little bFGF is found in the supernatant after centrifugation. At protein:heparin ratios of 12 or greater the bFGF in the supernatant increases as the protein:heparin ratio increases. These results suggest that the bFGF interacts with heparin to form complexes with hydrophobic surfaces. As the heparin is being saturated with bFGF (protein:heparin ratio of 8 to 10) the availability of ionic groups to interact with the polar solvent diminishes and the protein-heparin complexes precipitate.

Finally, the mitogenic activity of bFGF was assessed by measuring ^{3}H-thymidine incorporation into Swiss 3T3 fibroblast DNA in the absence or presence of heparin. The dose-response curves for bFGF or bFGF-heparin complexes (ratios 1, 10, 100) were found to be essentially identical (Fig. 5), suggesting that the heparin does not affect the response of the 3T3 fibroblasts to the growth factor. Similar results have been obtained by Saksela et al. (1988) regarding the effect of heparin on the induction of plasminogen activator in endothelial cells exposed to bFGF.

DISCUSSION

The inability of trypsin to degrade bFGF in the presence of heparin suggests that complex formation between the growth factor and the glycosaminoglycan may involve the interaction between the positively charged lysines and arginines of the protein with the negatively charged groups of heparin. The partial resistance of bFGF to chymotryptic degradation in the presence of heparin indicates, however, that the interaction also extends to the hydrophobic regions of the protein. Based on amino acid sequence information from a number of heparin-binding proteins, it has been suggested that clusters of basic amino acid residues and pairs and groups of basic and aromatic residues may be involved in the binding of heparin to these proteins (Schwarzbauer et al., 1983). Two such sequences, each consisting of a segment approximately five amino acids long, are present in bFGF (Sommer et

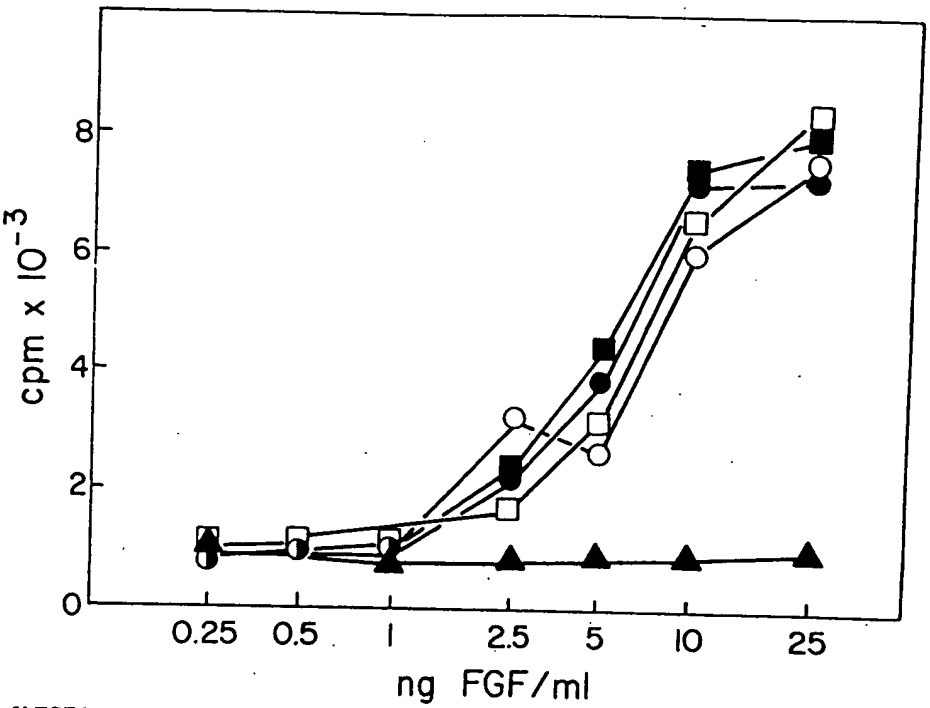

Fig. 5. Bioactivity of bFGF-heparin complexes. Samples containing bFGF alone (●); heparin alone (0.25–25 ng/ml) (▲); or bFGF-heparin complexes at ratios of bFGF:heparin = 1 (○), 10 (□), or 100 (■) were assayed for induction of ^{3}H-thymidine incorporation into Swiss 3T3 fibroblast DNA as described in Materials and Methods.

al., 1987; Esch et al., 1985), and they have been proposed to represent putative heparin binding sites of the growth factor (Gospodarowicz et al., 1987; Esch et al., 1985). Although these regions may function as the initial recognition sites for the protein-glycosaminoglycan interaction, the experimental evidence presented here suggests that the bFGF-heparin interaction affects a large portion of the bFGF molecule.

The physiological significance of the interaction of FGFs with glycosaminoglycans such as heparin or heparan sulfate is not clear. The major glycosaminoglycans are mostly present as components of proteoglycans (Hascall and Kimura, 1982) which are widely distributed in the extracellular matrix (ECM) (Heinegard and Paulsson, 1984). Cell-ECM interactions are known to influence a number of cellular processes including cell proliferation and morphogenesis. Indeed, FGFs are associated with the ECM (Baird and Ling, 1987; Vlodavsky et al., 1987) and possible mechanisms for the cellular mobilization of ECM-bound FGF have been discussed (Vlodavsky et al., 1987; Saksela et al., 1988; Moscatelli, 1987). The absence of a FGF signal peptide as deduced from the analysis of FGF cDNA and genomic clones (Sommer et al., 1987; Abraham et al., 1986a,b) leaves the question open as to how these mitogens are secreted from producer cells. The ECM proteoglycans by virtue of their high affinity for FGF, could act as "sinks" for the growth factor in areas of abnormal cell death or cellular injury and thereby prevent abberant cell proliferation and angiogenesis. In light of the possibility that the ECM could also represent a reservoir for FGF, one can speculate that the ECM-bound FGF is released by pericellular proteinases cleaving the protein core of proteoglycans, resulting in the generation of FGF-glycosaminoglycan complexes similar to those used in this work. Thus, the glycosaminoglycan portion of the complexes could serve as a carrier for the FGF molecules in a protease resistant form and allow for the diffusion of the growth factor through the surrounding ECM. Evidence that bFGF-glycosaminoglycan complexes can induce a positive response when exposed to sensitive cells has been presented here and elsewhere (Saksela et al., 1988; Moscatelli, 1987).

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EXHIBIT 4

Growth Factors

A Practical Approach

Edited by

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and

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Preface

There has been a major explosion in our interest in cytokines and their roles in regulating the function of the immune system in the past five to ten years. At the same time there has been a similar dramatic increase in research into the roles played by other growth factors in animal cell physiology. However, there are so many other factors (we have counted over 130—see *Appendix 1*) and they play so many roles that drawing them together as a group has proven quite difficult. Nevertheless we think we have got the balance between describing the detailed activities of individual factors and describing protocols which are appropriate to a wide range of factors just about right.

Although we have included the cytokines in our list of growth factors (*Appendix 1*), we have avoided protocols relating specifically to cytokines and this volume should be seen as complementary to the volume *Cytokines: A Practical Approach* in the same series.

We would like to thank all the contributors, especially those who got their manuscripts in by the deadline, and the staff past and present at OUP, who have been very pleasant and helpful throughout. We would also like to give special thanks to Paul Stroobant who helped tremendously with the list of factors in *Appendix 1*, and Mrs M. Cobbing who supplied many of the addresses in *Appendix 4*.

We would like to leave you with a final word of warning:

Growth factors are hard to purify and therefore expensive to buy. Details on how to look after your factors can be found in Chapters 3 and 6 but please read the manufacturer's instructions. We suggest you don't do what one researcher did to find out if he'd got his money's worth and run *all* your valuable recombinant factor on a polyacrylamide gel before fixing and staining it!

London
May 1992

IAN McKAY
IRENE LEIGH

Contents

NSB	non-specific binding
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDBu	phorbol dibutyrate
PFA	paraformaldehyde
PI	phosphatidyl inositol
PKC	protein kinase C
PMSF	phenylmethylsulphonyl fluoride
PTH	parathyroid hormone
RIA	radio-immuno assay
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SH-FBS	factor-free FBS
SPA	scintillation proximity assay
TBE	Tris-borate EDTA buffer
TBS	Tris-buffered saline
Tc	total counts
TCA	trichloroacetic acid
TEMED	*n*,*n*,*n*′,*n*′,-tetramethylethylenediamine
TESPA	3-aminopropyltriethoxy-silane
Tm	melting (dissociation) temperature
TPA	tetradecanoyl 12,13 phorbol acetate

1

Types of growth factor activity: detection and characterization of new growth factor activities

I. A. McKAY

1. Introduction

This book concerns itself with peptide molecules which transmit signals between animal cells. Despite the fact that these molecules may function as growth stimulators and/or growth inhibitors and that they may modulate differentiated functions of cells, they have come to be known collectively as *growth factors*. Many such factors have already been described (see *Appendix 1*), so it is the aim of this chapter to outline ways of characterizing a new factor with the emphasis on avoiding re-characterization of existing factors. Details of the individual methods involved will be covered in the following chapters or in other books of the Practical Approach series.

The all important aspect of characterizing a new factor is choice of assay system, and in this chapter the investigator will find suggestions for assay systems along with warnings about some of the pitfalls which may be encountered in their use. First, however, the various types of growth factor activity that the investigator may encounter are described.

2. Types of growth factor activity

2.1 Nature of the activity

Cells may communicate with each other directly through molecular interactions at the cell surface or by metabolic co-operation through gap-junctions. They may also interact indirectly by release of molecules, such as peptides or steroids, which can act locally or systemically to influence cell proliferation. Such growth factors can regulate cell proliferation in a positive manner (in which case they may also be referred to as *mitogens*—promoters of mitosis, i.e. somatic cell division), or in a negative fashion either directly or by induction of other factors. In many cases it is not feasible to dissociate effects of growth factors from their effects on differentiated cell function. For

Table 1. Potential growth factor interactions

GF's can stimulate or inhibit cell division, cell differentiation, and cell migration.

They can work with other factors in additive, co-operative, synergestic, or antagonistic fashion, but note that combinations can act in a fashion unpredictable from their originally defined activities.

GF's can act differently on different tissues/cell types, and can act differently at different concentrations, or at different times on the same tissue/cell type.

GF's are susceptible to antagonists, inhibitors, binding proteins, proteolytic degradation, soluble receptors, receptor up- or down-regulation, and mutations preventing their secretion.

GF's can up- or down-regulate transcription of other factors, and may up- or down-regulate their own transcription.

GF's can up- or down-regulate receptors for themselves or other factors.

Some GF's and/or their mRNA's are synthesized and turned over very rapidly.

They may induce mRNA or protein stabilization or degradation, and they may be susceptible to these phenomena themselves.

Some GF's are active when inserted in the cell's plasma membrane.

They may exist as inactive or partially active precursors which require proteolytic activation.

They may need to be bound to matrix molecules for their activity.

Some GF's need N-, O-, or both forms of glycosylation for their activity, and some require phosphorylation.

Some factors may need to form dimers or other oligomers to be active, and depending on the subunit composition, the bio-activity may even be opposite.

example some of the interleukins regulate T- or B-cell differentiation but are also potent mitogens serving as colony-stimulating factors. Likewise, growth factors may stimulate cell migration either in a specific chemotropic or chemotactic manner, or in a non-specific fashion as exemplified by scatter factor. Frequently a factor is identified as having a specific effect on differentiated cell function and is then found to be a mitogen in another system. Again using scatter factor as an example, it has been identified with hepatocyte growth factor—a positive regulator of liver epithelial cell growth.

Any cell exhibits receptors for a variety of growth factors and exposure to more than one of those factors at any time can lead to complex interactions and cell responses. *Table 1* lists some of the effects that growth factors can have on cells and some of the ways in which these effects can be modulated by other factors.

2.2 Modes of factor action

Five major modes of growth factor action have been postulated to date. In 1980 Sporn and Todaro suggested that all factor activities could be classified

into three: *endo*crine, *para*crine, and *auto*crine (1). Once it was discovered that factors inserted into the plasma membrane could stimulate replication of neighbouring cells, a fourth classification, *juxta*crine, was added to the list (2). A number of researchers have observed that factors which are made in cells but are incapable of being secreted or of being inserted into cell plasma membranes nevertheless induce observable phenotypic changes in those cells. The suggestion has been made that this represents an '*intra*crine' mode of action (3) whereby the factor interacts with its receptor in, for example, the golgi apparatus. A sixth mode of action, in which the growth factor must first be bound to a matrix molecule before presentation to its receptor on the cell surface has been demonstrated *in vitro* and could be regarded as a separate mode, but is perhaps best thought of as a sub-division of endocrine, autocrine, and paracrine modes. *Figure 1* shows the mechanisms involved in these different modes of action. Upon discovering a putative new growth factor, the discerning will investigate whether it has activity in each of these possible modes.

Tests on cultured cells, in particular the murine, Swiss, and Balb/c 3T3 cell

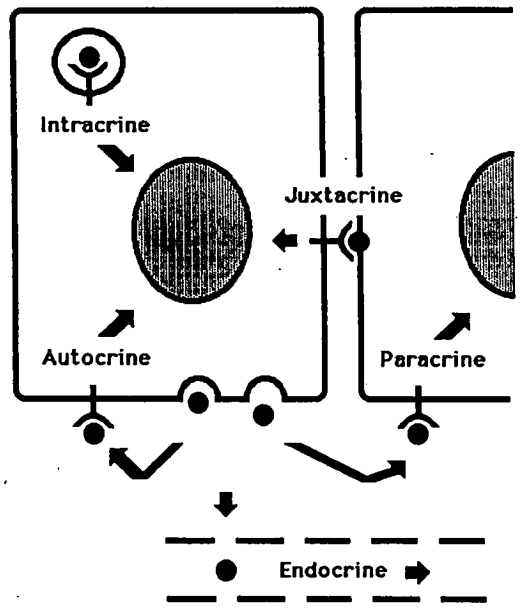

Figure 1. The various modes of growth factor activity are illustrated. Factors (black circles) are produced by cells and act on the cells producing them (autocrine, intracrine modes) or on other cells (paracrine, juxtacrine or endocrine modes), through specific receptors. The end result is shown as an arrow pointing to the cell nucleus, signifying stimulation or inhibition of cell division, cell differentiation or cell migration. In the case of the endocrine mode, factors are transferred in lymphatics or blood vessels to act on cells in distant parts of the body.

lines, have revealed that certain mitogens on their own will only stimulate a small percentage of cells to leave the quiescent Go state and enter the cell cycle. However combinations of factors will often show synergy in their ability to induce cell division and this has led to the concept of *competence* factors which initiate the move from quiescence, and *progression* factors which allow cells to proceed to division. This concept is further explored in Chapter 3. Suffice it to say this further classification of growth factor activities should be borne in mind when investigating the nature of a new factor.

3. Known growth factors

We have counted at least 20 different growth factor families including as many as 130 different individual factors, and it is likely that many more will be discovered or synthesized before this book is published. It is important therefore for the investigator who has just identified a new activity to be aware of the major existing families so that he or she does not reproduce previous work unnecessarily.

Appendix 1 lists many of the known growth factors in superfamilies and families. It should be noted that the designation 'family' implies structural homology between the various members and so the interleukins, which share a common designation without any structural basis, have not been lumped together as a family. It is not the remit of this book to provide definitive information on the structure and function of all these factors and their receptors. Instead the investigator is referred to the many excellent reviews of different factors which may be found in the literature. In particular the role of factors in different tissues have been detailed in a number of relevant volumes of the Annual Review series (see 4–8).

4. Choice of source material containing putative factors

In the search for an entirely new growth factor, there is no limit to the range of starting materials one might employ. They may be synthetic or organic, and the organic ones may be physiologically relevant to their source organism or they may be derived from entirely unrelated species. Peptide libraries (9, 10) offer a further, fertile source of new agonists and antagonists for growth factor receptors and binding proteins. Here we list some of the alternative materials with which the investigator might start the search for a new factor.

4.1 Whole organism, organ, tissue, or cell preparations

In the past researchers have taken whole organisms, organs, tissues, or cells and puréed them in homogenizers, blenders, and the like. The resulting paste can be diluted in balanced salt solutions and assayed in various test systems.

Obviously this method, when applied to whole organisms is limited by the size of the organism; getting an extract of whole elephant would, we imagine, be problematical for even the most diligent of researchers. However this method has been used on whole *Xenopus* or mouse embryos and, for example, in extracting an activity from cultured keratinocytes which has been shown to stimulate wound healing *in vivo* (11).

Such preparations are quite rightly known as crude homogenates and the investigator is warned that this approach may lead to laborious re-identification and characterization of a known factor.

4.2 Conditioned medium from cultured cells

Cultured cells, and in particular cell lines, can provide a useful homogeneous source, rich in potential for the isolation of new factors. Again however the investigator should take care that the cell population is indeed homogeneous, particularly in the case of primary cultures. Often these cultures will be contaminated with other cell types, the ubiquitous fibroblast for example, making it extremely difficult to identify the source of a new activity.

A wide variety of cell lines and strains are held in the American Type Culture Collection and in the European equivalent, the ECACC at Porton Down. *Appendix 2* gives addresses for these repositories.

Other chapters in this book and in several other books in the Practical Approach series, including *Cell Growth* and *Tissue Culture Methods*, give details of how to isolate cells from tissues and how to grow cells in culture.

4.3 Other sources

4.3.1 Toxins

The massive inflammatory response occasioned by bacterial infections, stings, and bites suggests potent effects of toxins on cell metabolism. Indeed a substantial number of cell mitogens have been isolated from these sources—cholera toxin, *Pasteurella multocida* toxin (PMT) and Mastoparan, for example. *Appendix 1* lists some of them but there can be little doubt that many remain to be characterized.

4.3.2 Secretions

Unlike insects with their stings, other animals may concentrate their defence molecules in secretory glands. Bombesin has been isolated from frog skin and presumably this source awaits exploitation in other amphibian species. However not all secretions contain defence molecules; epidermal growth factor (EGF), for example, was found to be secreted from mouse salivary gland.

4.3.3 Synthetic factors

Synthetic factors is a catch-all title which includes all recombinant factors. Clearly the 'diet' or un-glycosylated form of a factor expressed in bacteria

may have a substantially different activity (including no activity) when compared to its homologue, correctly expressed in its native tissue. Other types of synthetic factor include those which are hybrids of more than one factor.

In the search for new activities many companies have put efforts into mutation of existing factors or, in the case of small peptides, have investigated the effects of substitutions, abbreviations, and elongations. The search for new peptides has been taken to extremes in the generation of libraries which have millions, or even billions, of peptides with more or less random sequences (9, 10).

Synthetic factors also include those non-peptide factors which have effects on cell metabolism. An example of such a factor would be dibutyryl cAMP which, like cholera toxin, can be a potent cell mitogen in the correct circumstances. Other commonly used non-peptide growth factors are listed in *Appendix 1*.

5. Identification of new factors

Once a putative new growth factor activity has been discovered, it is necessary to characterize this activity further *in vitro* and *in vivo*. *Figure 2* outlines a protocol which will help the investigator to avoid re-characterization of a known factor. One of the more important aspects of this procedure is the assay of activity.

5.1 Choice of assays of factor activity

The first aim of the investigator should be to keep the number of assays to a minimum. This can be achieved by using only those which are pertinent. Clearly assays of neural regeneration are not initially relevant to the characterization of a factor which inhibits the growth of endothelial cells, whereas an assay of angiogenesis might be essential. One should always be aware of the restrictions of the *in vitro* assay, for example, measuring the stimulation of keratinocyte proliferation *in vitro* covers only a limited aspect of wound healing promotion *in vivo*. Some of the alternatives for assays of growth factor activity and some of the factors which may influence the investigator's choice are now presented.

5.1.1 *In vitro* assays of cell division

When searching for new growth factors it is useful to test their effects on cells which have a wide range of receptors and second messenger systems such as mouse 3T3 cells (Chapter 3). Having determined whether the factor or factors stimulate or inhibit 3T3 cell proliferation, the investigator may wish to test their effects on proliferation of other cell types such as embryonic (Chapter 5), epithelial (Chapters 2 and 6), neural (4), or haemopoietic cells (12).

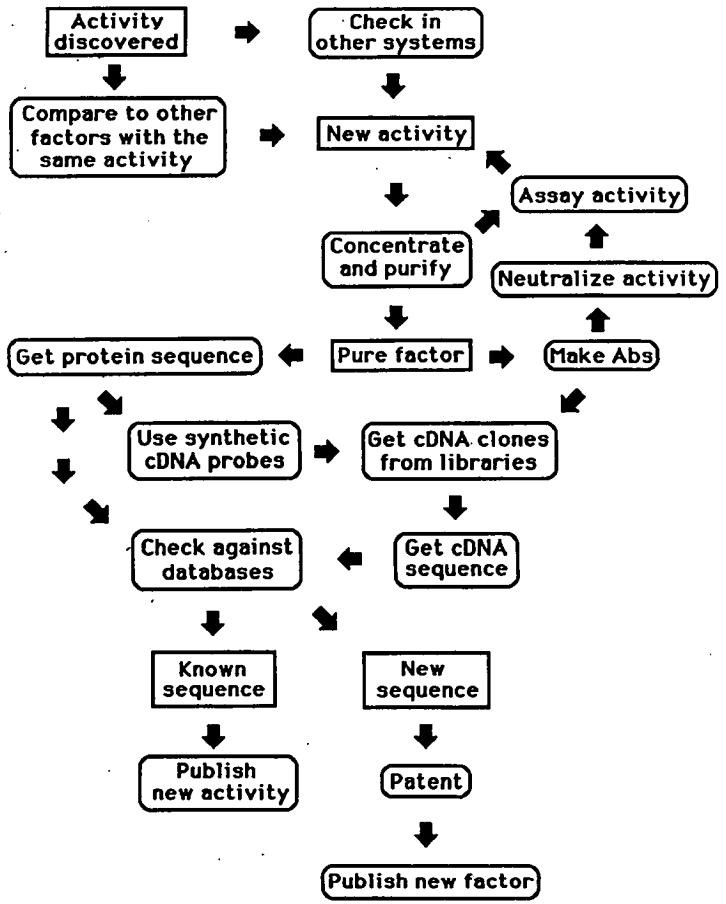

Figure 2. Protocol for the characterization of putative new growth factor activities.

It is of course always possible that the factor will have only a small effect on cell proliferation either way. In this case the investigator should ask:

(a) Does the target cell have enough receptors?

(b) Is an effect on cell proliferation masked by the presence of too much serum (see Chapter 2)?

(c) Does the preparation contain an inhibitor of proliferation in addition?

(d) Does the factor require nutritional factors such as transferrin or insulin for its activity?

(e) Has the result of the assay been read at the optimal time after the addition of the factor?

(f) Does the factor require modification or another growth factor for its activity? (In this case the investigator is referred to *Table 1* for some of the possible pitfalls in the assay of growth factor activity.)

Demonstration that a particular factor is causally involved in regulation of cell division etc. usually requires specific intervention in the signalling pathways activated by that factor (13). Methods of intervention include the use of cells lacking particular receptors (14), mutated receptors, receptor antagonists, neutralizing antisera, and antisense strategies (see Chapter 3 for a more complete discussion of this topic).

5.1.2 *In vivo* assays of cell proliferation

Growth factors play an important role in regeneration of tissues, organs, and even whole limbs. This often involves the interplay of a number of different cell types in division, migration, inflammation, and matrix re-modelling. Each of these processes can be influenced by a number of different growth factors. However, at a gross level, it is sometimes possible to pick out the role of individual factors. For example TGF-α is known to play an important role in epidermal wound healing, and TGF-β is involved in regulating bone growth and repair (see Chapter 6). Several animal models exist for assay of the effects of growth factors on regeneration of muscle, nerves, bone, liver, skin, and tissue capillary supply (15).

The investigator is reminded that growth factors may show species specificity in their activities on any given tissue.

Certain growth factors, in particular the cytokines, have potent effects on tumour cell growth and these effects are commonly investigated in syngeneic immunocompetent or in allogeneic immunodeficient animals. One method involves getting expression of the factor in the tumour cells and then determining the effect of the factor on tumour growth (16). Other uses of this system include testing the effects of antisense polynucleotides on growth factor expression and cell proliferation, or inactivation of the growth factor's gene by homologous recombination.

Some animal strains may be mutant in expression of a particular factor or its receptor. Thus the gene encoding the ligand for the c-kit receptor (itself encoded by the white spotting locus, *W*) was found at the steel locus (*Sl*) (17) and is now called mast cell growth factor (MGF) (18). Such animal strains constitute unique test systems for investigating the effects of factors on cell proliferation and tissue formation.

Embryos can be extracted from animals, modified, and replaced. Modification of the genome results in the production of transgenic animals. Modification with growth factor genes under the control of different promoters, or inactivation of endogenous growth factor expression, will prove a powerful system for the investigation of the role of factors in tissue and organ development.

5.2 Purification of the factor

Once a putative novel growth factor activity has been identified it is important that the active element be purified for identification. Many peptide growth factors are active in picomolar or even femtomolar concentrations and are often scarce in their source material. It is therefore usual to concentrate these factors as an early step in their purification and this procedure is detailed in Chapters 2 and 9. At later stages of the purification procedure it is necessary to check that the material maintains its biological activity. At the same time strenuous efforts should be made to ensure that the newly purified factor has not already been characterized. A simple check list is useful at this point.

(a) Does the factor share the activity with any known factor?

(b) Does the factor compete with a known factor for its receptor?

(c) Do antisera to known factors neutralize the new activity?

If the answer to any of these is yes then further assay of the newly purified factor in other systems should be avoided until sequence data are available to confirm or disprove factor identity.

5.3 cDNA cloning and sequencing

Once sufficient purified material is available (usually microgram to milligram quantities), attempts may be made to clone the gene or genes encoding the factor. Two routes are usually followed.

(a) Use antibodies to the factor to isolate the corresponding cDNA from an expression library (see Chapter 8).

(b) Get some peptide sequence data and generate a synthetic oligonucleotide probe which can be used to screen cDNA libraries for related sequences.

While the second seems more straightforward, the number of places where one can get peptides sequenced reliably are few and most good sequencers have enough work to keep them going for years!

5.3.1 Sequence identity

With some peptide or cDNA sequence data it is important once again to check whether the factor is previously unreported. Note that factors may share N- or C-terminal sequences and still not be identical. It is also recommended that the cDNA sequence should be lined up with the corresponding genomic sequence. Some libraries may contain scrambled cDNA clones. A further word of caution here. Many sequences are first reported in patent applications and it is worthwhile searching patent databases in addition to the more usual sequence repositories.

6. What to do with a new growth factor

First patent it! Many companies are willing to buy the rights to new factors and even if the factor has no immediately obvious application, it may prove to have one in the future. Under current legislation in the UK it is essential to submit a patent application before disclosing any pertinent data into the public domain. In other parts of the world the situation differs. However, if in doubt, do not disclose any information to anyone, other than your immediate colleagues, especially if outsiders are present, as this may be construed as disclosure into the public domain. This includes references in abstracts or posters submitted to meetings. Caution your colleagues to maintain a strict silence too. Future revenue may depend on it!

Next, and only after patenting, publish it.

Table 2 outlines other possible uses of a new growth factor and its cloned gene.

Table 2. Common uses for growth factors, their cloned genes, and cDNAs

Protein
May be used in analysing pathways of mitogenesis and/or inhibition of cell growth (see Chapters 2, 3, 5, and 8).
Can be used alone or conjugated to toxins etc. for therapeutic purposes (see Chapter 6).
Make antibodies to the protein for immunoassays and immunocytochemistry (see Chapters 10 and 11).
Derivatives of anti-factor antibodies with radiolabels, toxins, etc. may be used for diagnostic and therapeutic applications.
cDNA
Use the cDNA to get expression of the factor from bacteria or eukaryotic cells in large amounts (see Chapter 9).
Use the cDNA as a probe for factor expression in northern blots.
Use the cDNA as a probe for the tissue distribution of the factor by *in situ* hybridization analysis (see Chapter 7).
Use the cDNA to look for related genes in cDNA or genomic libraries from the same or different tissue/species.
Put the cDNA under the control of a different promoter for expression in other cell types *in vitro*.
Use the cDNA to get tissue-specific factor expression or ablation of expression in transgenic mice.
Make antisense cDNA constructs to inhibit factor expression *in vitro* or *in vivo*.
Genomic clone
Investigate intron/exon structure and compare with other known factors.
Look for potential regulatory sequences and analyse them.
Use RFLP's in diagnosis.

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2

Detection of polypeptide growth factors: application of specific bio-assays and PCR technology

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1. Introduction

The term 'growth factor' is used to describe a polypeptide produced by a cell or isolated from a tissue, and having a growth-regulatory role on other cell types. This activity is usually discovered by application of *in vitro* bio-assays which quantitate the dose-dependent factor-mediated growth modulation of a homogeneous culture of a particular target cell. However, some peptide growth factors both stimulate and inhibit cell proliferation and some may have biological effects which are unrelated to cell growth, such as the differentiation of certain cells. The first phenomenon can be due to the identity of the target cell used in the bio-assay, the concentration of the factor tested (growth-stimulatory versus growth-inhibitory concentrations), and the presence in the growth-factor preparation of other polypeptides which may act synergistically or antagonistically with the growth factor under study (see *Table 1*, Chapter 1).

Endocrine hormones and cytokines (regulatory proteins secreted by cells of the immune system) are mostly considered as a separate class of important polypeptides. It is now becoming clear that growth factors and cytokines have a broader range of action than their original name suggests. This means that many of these polypeptides of which the activities have been defined in different *in vitro* systems, and therefore have received a name according to this activity, are in fact identical molecules. Different polypeptides may also share biological activities (at least *in vitro*), some growth factors are expressed as a secreted and as a membrane-bound polypeptide, and—last but not least—some polypeptide growth factors may even share the same receptor (such as epidermal growth factor and transforming growth factor alpha). Therefore, all classifications are somewhat arbitrary and one should strongly consider these polypeptides not just as growth factors, despite this name

being the most commonly used (including in this volume), but rather as multipotent molecules.

Growth factors, just as for example interleukins, are involved in many physiological processes and will possibly find therapeutic and diagnostic applications in a wide variety of fields. However, it is our feeling that many experimental tools and protocols which are available for interleukins (such as neutralizing antibodies and specific *in vitro* assays using cell lines which are interleukin-dependent) are not generally available for growth factors. All this may make it difficult for research groups to take the decision to enter the field. This chapter, like the others, aims at helping to overcome this block. It describes the phenomenon of growth factor dependence, with particular reference to insulin-like growth factor activity in epithelial cells, and proceeds to describe sensitive detection of growth factor mRNA expression by PCR technology in keratinocytes and other epithelial cell types. This chapter therefore complements Chapter 3 in which the effects of growth factors on fibroblasts are described.

2. The concept of growth factor-dependence

2.1 Growth factor-dependence of cellular proliferation

Mammalian cells require a variety of nutrients for optimal maintenance and survival *in vitro*. Substrate-attached cells may require additional attachment proteins, but even together these nutrients and attachment factors are generally not sufficient for induction of cell proliferation. This latter process requires the additional presence of externally added polypeptide growth factors, particularly in the case of non-transformed cells. Tumour cells often have a reduced requirement for externally added polypeptide growth factors for proliferation. This may be caused by the fact that transformed cells themselves generally produce such growth factors, to which they can respond in an autocrine manner. Alternatively, transformed cells may proliferate in the absence of growth factors as a result of activation of cellular proto-oncogenes (1).

Polypeptide growth factors are traditionally sub-divided into two major categories, the cytokines or haematopoietic growth factors, and the growth factors for substrate-attached cells. Although this division is artificial, haematopoietic growth factors generally have a highly specific function in proliferation, differentiation, and survival of haematopoietic cells. Growth factors for substrate-attached cells act on cells of most solid tissues in the body, and in contrast to most cytokines, receptors for this category of growth factors often have intrinsic tyrosine phosphokinase activity. However, certain cytokines may also act through this class of receptor molecules, while in addition a number of cytokines may act on substrate-attached cells. Moreover, members of the transforming growth factor β (TGF-β) family, which are generally categorized as growth factors for substrate-attached cells, also act on haematopoietic cells and exert their action by receptor molecules which lack tyrosine

phosphokinase activity (2). Expression cloning of the activin (a member of the TGF-β family) and TGF-β type II receptor has identified these receptors as serine/threonine kinases (3, 4). The application of polymerase chain reaction (PCR) technology has allowed identification of alternatively spliced mRNAs of a second activin receptor gene coding for other transmembrane activin receptors which are related to the receptor coded for by the first gene (5).

The growth factors for substrate-attached cells were originally divided into five major families, namely epidermal growth factor (EGF), TGF-β, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and the heparin-binding growth factor (HBGF) families. The number of growth factors in each family has rapidly expanded in recent years, and not all factors can be categorized now in one of the originally proposed families (6) (see *Appendix 1*).

In order to study the cellular requirements for externally added polypeptide growth factors for proliferation, culture conditions should be applied that allow variation of the amount and nature of growth factors added, while maintaining optimum concentrations of nutrients and attachment factors. Fetal bovine serum (FBS) is generally added as a supplement to culture media for the induction of cell proliferation, but serum is not only a good source of polypeptide growth factors, it also contains valuable nutrients and attachment factors. Serum-free media have been designed for a variety of individual cell lines, but no single serum-free medium is available that is suitable for a variety of different cell lines in culture. Following the observation that growth factors generally require intact disulphide bridges for activity, we have shown that growth factor activity in serum can be inactivated by treatment with dithiothreitol, followed by iodoacetamide (7). The preparation thus obtained (see *Protocol 1*) has been designated growth factor-inactivated serum (SH-FBS). It still contains a variety of maintenance factors for cells in culture, including lipids, protease inhibitors, and cell attachment proteins, but is devoid of growth-stimulatory activity. Thus, a culture medium can be applied which consists of a rich nutrient base such as a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (further referred to as DF medium), with 10 μg/ml transferrin and 30 nM Na_2SeO_3. When this so-called DFST medium is supplemented with 0.2% bovine serum albumin (BSA) and 10% SH-FBS, it provides optimum conditions with respect to nutrients and attachment factors, but is devoid of growth-stimulating activity. Under these growth factor defined culture conditions, selected polypeptide growth factors can now be added at required concentrations (7).

Protocol 1. Preparation of growth factor-inactivated serum (SH-FBS)

You will need the following buffers:

- 100 × phosphate buffer: 24 g KH_2PO_4, 172.8 g $Na_2HPO_4.2H_2O$, add water to final volume of 1.2 litres (pH 7.3)

Protocol 1. *Continued*

- PBSØ: 80 g NaCl, 2 g KCl, 100 ml phosphate buffer 100 ×, adjust volume to 10 litres with distilled water (pH 7.3)
- PBS: add 9 ml of a 1 M $CaCl_2$ stock, and 5 ml of a 1 M $MgCl_2$ stock to 10 litres of PBSØ

Day 1

1. Add 7.7 g of dithiothreitol to a bottle containing 500 ml of fetal bovine serum (FBS; not heat-inactivated).
2. Stir for 2 h at room temperature (yields a turbid solution).
3. Cut two pieces of dialysis tubing (exclusion limit 8–15 000 d), and treat them according to the manufacturer's instructions.
4. Fill the tubes with the serum.
5. Dialyse tubes overnight at 4°C in one bucket (10 litres) of PBSØ each.

Day 2

6. Dialyse both tubes together in one bucket of PBSØ for 5–6 h.
7. Pour the serum in a bottle and add 2.5 g of iodoacetamide.
8. Stir for 2 h at room temperature.
9. Pour the serum (without foam) in the thoroughly washed dialysis tubes.
10. Dialyse the serum overnight in two buckets of PBSØ.

Day 3

11. Continue to dialyse the whole day in one bucket of PBSØ.
12. Dialyse further overnight in two buckets of PBSØ.

Day 4

13. Dialyse the whole day further in one bucket of PBSØ.
14. Finally, dialyse the serum overnight in two buckets of Ca^{2+} and Mg^{2+} containing PBS.

Day 5

15. Centrifuge at 10 000 *g* for 30 min at 4°C (yields a large pellet).
16. Centrifuge the supernatant at 100 000 *g* for 60 min at 4°C.
17. Collect the supernatant (SH-FBS), and sterilize by filtration.
18. To remove remaining steroids, the SH-FBS can be treated with dextran-coated charcoal (see *below*) to yield DCC-SH-FBS. This is however not essential for the IGF bio-assay.

Using growth factor defined culture conditions, normal rat kidney (NRK) cells, a non-transformed fibroblast cell line, become quiescent in the absence of externally added polypeptide growth factors. In the presence of optimum concentrations of EGF and insulin, however, these cells proliferate at least as rapidly as in conventional serum-containing media (7). In contrast, simian-sarcoma virus (SSV) transformed NRK cells proliferate as rapidly in growth factor defined medium as in media supplemented with normal fetal bovine serum, i.e. even in the absence of externally added growth factors (8). This is in line with the observation that SSV-NRK cells themselves produce growth factors from virtually all five of the above families, and they can respond to these factors in an autocrine manner (8). Using this strategy we have shown that non-tumourigenic cells generally become quiescent in the absence of polypeptide growth factors, while most tumour cells are not dependent on externally added growth factors for proliferation. This has been shown particularly clearly for several embryonal carcinoma cell lines of both murine and human origin (9, 10).

2.2 Growth factor-dependence of human MCF-7 breast cancer cells

Notable exceptions to the general observation that tumour cells do not require externally added polypeptide growth factors for proliferation, are oestrogen-dependent human breast cancer cells, in particular the MCF-7 cell line (11). *Figure 1* shows a time-course for proliferation of MCF-7 cells in various culture media. Under growth factor defined assay conditions these cells are unable to proliferate, unless specific growth-stimulating hormones, such as insulin and oestradiol (E_2) are added. In the presence of these factors, however, cells proliferate as rapidly as in normal serum-containing media. These assays have been carried out in media devoid of phenol red, since it is known that this pH indicator is a weak oestrogen receptor agonist. Moreover, the serum samples tested were first treated with dextran-coated charcoal to remove endogenous oestrogens (see *Protocol 2*). Analysis of Hoechst stained DNA by means of a fluorescence activated cell sorter confirmed that in the absence of growth factors MCF-7 cells accumulate in the G_1/G_0-phase of the cell cycle, as non-transformed cells do (11).

Protocol 2. Preparation of steroid-stripped serum (DCC-FBS)

Day 1

1. Prepare dextran-coated charcoal (DCC) suspension. Add 900 ml of H_2O to 100 ml of Tris–HCl (0.1 M, pH 8.0). Dissolve 0.25 g of dextran (T500) in this solution, and add 2.5 g of charcoal (activated, untreated powder. Do not expose to air for longer periods; it may become contaminated).
2. Stir overnight at 4°C in tightly locked vessel.

Protocol 2. *Continued*

Day 2

3. Heat 200 ml of FBS for 30 min at 56°C in a water-bath.
4. Fill eight (plastic) 50 ml tissue culture tubes with DCC suspension and lock tubes.
5. Centrifuge in swing-out rotor for 20 min, 1000 *g*.
6. Discard supernatant.
7. Refill tubes with additional DCC suspension, and repeat centrifugation procedure.
8. Add heat-treated FBS to four DCC pellets and transfer to flask.
9. Incubate in water-bath at 45°C for 45 min, while shaking.
10. Refill the centrifugation tubes.
11. Centrifuge for 20 min at 1000 *g*.
12. Repeat this procedure with the four remaining DCC pellets.
13. Centrifuge remaining coal away from the serum before sterile filtering.

From the quiescent state, MCF-7 cells can be re-stimulated to proliferate by addition of defined growth factors (see *Figure 2*). The data presented here show that EGF, TGF-β, and PDGF by themselves do not stimulate growth of these cells even when added at elevated concentrations. HBGFs such as basic fibroblast growth factor (bFGF) are also unable to induce proliferation of quiescent MCF-7 cells (data not shown). Oestradiol by itself has only weak mitogenic activity, in contrast to insulin which is a potent stimulator of MCF-7 cell proliferation. Even when added in combination with other factors, no growth-stimulatory effect was observed by EGF or PDGF, while a slight inhibitory effect was observed when insulin or E_2 were added in the additional presence of TGF-β. These studies show that when added at sufficiently high concentrations, E_2 does not affect insulin-induced growth stimulation. Additional studies have shown, however, that E_2 has a significant effect on the dose-response curve of insulin-induced proliferation of MCF-7 cells, such that half-maximum growth stimulation by insulin occurs at 250 ng/ml in the absence, and at 5 ng/ml in the additional presence of E_2 (11). Direct effects of E_2 on proliferation of these cells can therefore particularly be observed in the presence of sub-optimum concentrations of insulin. Addition of saturating concentrations of insulin, however, by-passes the oestrogen requirements of MCF-7 cell proliferation completely.

3. Polypeptide growth factors of the insulin-like growth factor family

The above data show that insulin is the major mitogen for the oestrogen-dependent breast cancer cell line MCF-7. Together with insulin, the insulin-

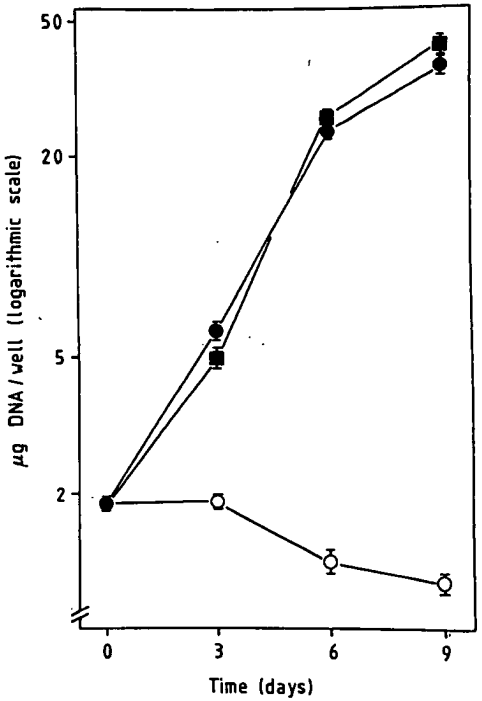

Figure 1. Growth factor dependence of MCF-7 cell proliferation. Cells were plated in phenol red-free medium containing 5% DCC-FBS, as described in *Protocols 2* and *3*. After a 24 h incubation period in DFST 0.2% BSA, the medium was replaced with DFST 0.2% BSA, now supplemented with 10% FBS (■—■), 10% DCC-SH-FBS (○—○), or 10% DCC-SH-FBS containing in addition 10 µg/ml insulin, 100 ng/ml EGF, and 10^{-9} M E_2 (●—●). Media were changed every three days. The increase in cell number was determined on the basis of cellular DNA content, as described in *Protocol 3*.

like growth factors (IGFs)-I and -II form a family of structurally related peptides, which play an important role in the control of cell proliferation as well as having a regulatory function in cell metabolism (12). All three proteins have a molecular size of 6–7 kd in their mature form. Larger, biologically active precursor forms have been described as well. IGF-I levels in the body, which are under stringent control of growth hormone, are directly associated with skeletal growth during puberty, while IGF-II is believed to play an important role during embryogenesis. In addition, IGFs are secreted by a variety of cells in different tissues, where they may play an important role in autocrine and paracrine processes.

All three growth factors have their own specific plasma membrane receptor, which in the case of insulin and IGF-I contains an intrinsic tyrosine phosphokinase activity, while the IGF-II receptor is identical to the 215 kd cation-independent mannose 6-phosphate (Man6-P) receptor (13, 14). There

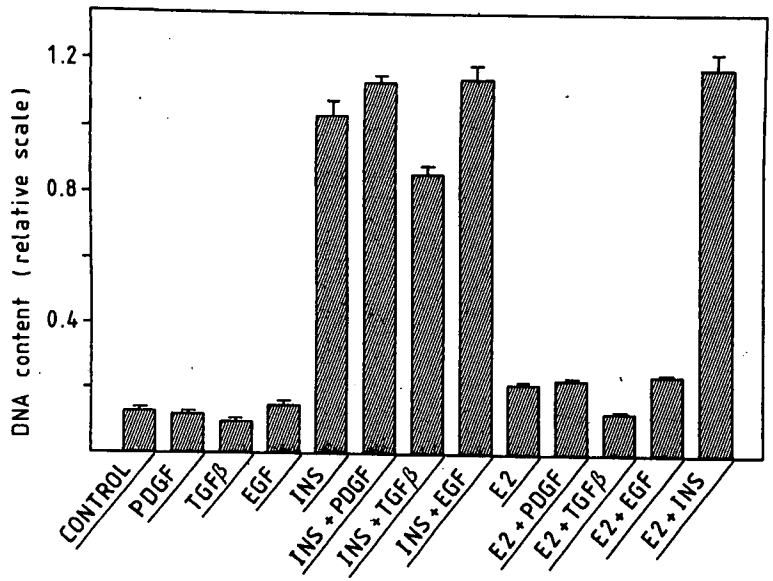

Figure 2. Stimulation of quiescent MCF-7 cells by growth factors. Quiescence was induced as described in *Protocol 3*, and the cells were stimulated by incubation in DCC-SH-FBS with the following additions: 10 ng/ml human platelet PDGF, 1 ng/ml $TGF\beta_1$, 100 ng/ml EGF, 10 μg/ml insulin (INS), and 10^{-9} M oestradiol (E_2). Cell number increase was determined on the basis of the DNA content after a four-day incubation period, and expressed relative to DNA content measured in cultures stimulated by 9% FBS.

are however only a few reports that suggest that binding of IGF-II to the Man6-P/IGF-II receptor is of biological importance. Binding of the ligand at the cell surface results in internalization and degradation of this ligand, but one critical question remains whether IGF-II binding to the Man6-P/IGF-II receptor results in signal transduction. This has been difficult to assess experimentally, since all three factors show considerable cross-reactivity with receptor molecules for other members of the IGF family. In particular both insulin and IGF-II can bind the IGF-I receptor with low affinity. In general, signal transduction mediated by the IGF-I receptor is believed to be associated with regulation of cell proliferation, while the insulin receptor predominantly mediates metabolic control. Recent considerations indicate that the IGF-II receptor most probably only has a scavenger function.

IGFs are generally present in the body in association with specific binding proteins. These IGF-binding proteins, of which various types have been identified, strongly enhance the lifetime of IGFs in the circulation. IGF-binding proteins may also be secreted by cells in culture, where they may either obscure or potentiate the biological activity of bound IGFs (15).

4. Biological assay for detection of IGF activity

The observation that insulin is a potent mitogen for quiescent MCF-7 cells when added in the μg/ml range, suggests that the growth stimulating activity of insulin is mediated by the IGF-I receptor. This is confirmed by the data shown in *Figure 3*, which represent dose-response curves of all three IGFs for induction of MCF-7 proliferation in the additional presence of oestradiol (16). Half-maximum stimulation was induced by human IGF-I at a concentration of 0.6 ng/ml, by bovine insulin at 5 ng/ml, and by human IGF-II at 10 ng/ml. These data are in agreement with the above hypothesis that growth stimulation of MCF-7 cells is mediated by the IGF-I receptor, although the relatively high sensitivity for bovine (and also human) insulin suggests that the intrinsic insulin receptor in this cell line may also contribute to the cellular growth control (17).

MCF-7 cells can be selectively stimulated to proliferate by members of the IGF family, and this without interference of growth factors from other families. This implies that the present assay can be used as a selective bio-assay for detection of IGFs (6). When carried out in the presence of E_2, this assay is particularly sensitive for IGF-I, with a detection limit of approximately 0.2 ng/ml. For the other two members of the IGF family detection limits are of the

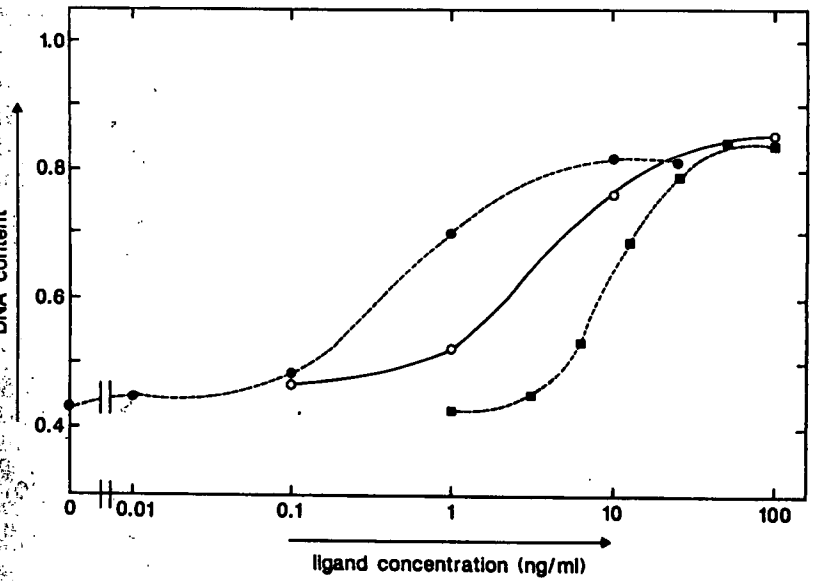

Figure 3. Dose–response curves for growth stimulation of quiescent MCF-7 cells by pure human IGF-I (●—●), bovine insulin (○—○), and human IGF-II (■—■). The assays were carried out as described in *Protocol 3*, in the additional presence of 10^{-9} M E_2. Data are expressed relative to stimulation by addition of 9% FBS, while background levels correspond to induction by E_2 alone.

order of 2 ng/ml for insulin, and 5 ng/ml IGF-II. The high sensitivity of these cells for IGF-induced stimulation suggests that MCF-7 cells may produce IGF-binding proteins themselves (18), but that the amounts produced do not significantly interfere with the activity of externally added IGFs. Members of the TGF-β family interfere slightly with this assay, since they may reduce the extent of IGF-induced growth stimulation, but under no condition was a complete repression of IGF-induced proliferation by TGF-β observed (11). A variety of other growth controlling molecules, such as histamine, bombesin, prostaglandin F2α, bradykinin, thrombin, and carbachol, were also without effect in this assay. Tumour promoters such as tetradecanoyl 12, 13 phorbol acetate (TPA), however, completely blocked IGF-induced proliferation, which is caused by an irreversible differentiation of these epithelial cells into non-proliferating derivatives. The exact procedure used for this specific bio-assay is described below (see *Protocol 3*).

Protocol 3. Bio-assay for insulin and IGFs

Cell culture

The MCF-7 cells used for this assay are cultured at 36.5–37°C in phenol red-containing DF medium (obtained from Gibco), supplemented with 7.5% FBS, and buffered with 44 mM sodium bicarbonate, in a humidified atmosphere containing 7.5% CO_2. They are passaged twice weekly using trypsin (0.05%) and EDTA (0.02%) in order to maintain them in an exponential growth phase. Cells to be used in the bio-assay were plated at a density of 10^4 cells/cm^2 in the above serum-containing medium, and grown for four days (pre-culture). All subsequent incubations of the cells were carried out at 36.5–37°C. The period of time outside the incubator should be kept short. Variants of the MCF-7 cell line that have a different sensitivity to mitogens exist. The cell line used here is available on simple request.

Day 1

1. Trypsinize the MCF-7 pre-culture.
2. Pass the cells three times through a thin needle to obtain a suspension of single cells and count the cells.
3. Plate 1.5×10^4 MCF-7 cells per well in a 24 well tissue culture plate (1.8 cm^2) in 1 ml of phenol red-free DFST medium (DF with 30 nM Na_2SeO_3 and 10 μg/ml transferrin), containing 0.2% BSA (fraction V), and 5% DCC-FBS.

All subsequent incubations are carried out in phenol red-free media.

Day 2

4. Replace medium by 1 ml per well of DFST containing 0.2% BSA.

Protocol 3. *Continued*

Day 3

5. Prepare test samples.
   (a) Conditioned medium can be used directly in 100 μl aliquots.
   (b) If necessary, dilutions of test samples are made in BES (2-[bis[2-hydroxyethyl]-amino] ethane-sulphonic acid; 50 mM, pH 6.8)-buffered DFST supplemented with 0.2% BSA.
6. Replace medium in wells by 900 μl of DFST supplemented with 0.2% BSA, 10% SH-FBS (or DCC-SH-FBS), and 10^{-9} M 17β-oestradiol.
7. 24 h after medium change at day 2, add 100 μl test sample per well (in triplicate). If sample volumes are larger than 100 μl, the concentration of SH-FBS and oestradiol in the above medium should be adjusted so that the final concentrations remain constant.
8. Add 100 μl of FBS to three wells on each plate as a positive control (final concentration of serum in the assay is then 9%).

Day 7

9. Wash the cells gently with PBS (only once to prevent cell loss).
10. Store the dry plates at −20°C before measuring total DNA content of cells.
11. Perform the DNA measurement.
    (a) Add 2 ml of buffer (0.04 M Na_2HPO_4, 0.01 M NaH_2PO_4, 2.0 M NaCl, 0.002 M EDTA, pH 7.4) containing 1 μg/ml Hoechst 33258 (stored as a 500 × concentrated stock) to each well.
    (b) Sonify for 10 sec to suspend the cells.
    (c) Transfer this suspension to a cuvette and measure the fluorescence in a fluorometer (excitation 356 nm, emission 458 nm).
    (d) Express the DNA content relative to the value measured in 9% FBS according to Labarca and Paigen (1980) *Anal. Biochem.*, **102,** 344.

Tissue extracts or serum-free media conditioned by cells in culture can be assayed directly for IGF bio-activity in this assay (19, 20). The net activity measured may well result, however, from the concomitant presence of IGF-binding proteins in these samples. IGF-binding proteins have molecular sizes above 40 kd (15). Therefore, these proteins can be separated from IGFs by gel chromatography in a low pH buffer (16, 19). *Figure 4* shows the elution pattern of serum-free conditioned medium of P19 EPI-7 cells, a differentiated murine embryonal carcinoma cell line, on a Bio-Gel P-100 column in 1 M acetic acid. This cell line shows high expression of the IGF-II gene, but produces in addition a variety of other growth factors, including PDGF-AA and type β TGFs (16).

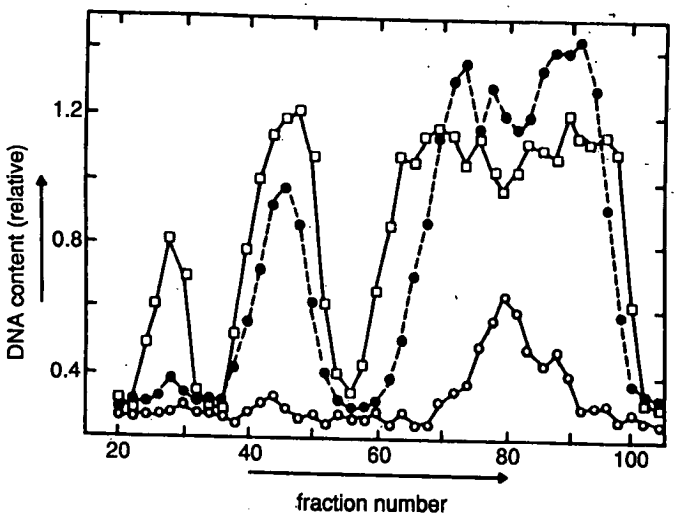

Figure 4. Elution profile of IGF biological activity secreted by P19 EPI-7 cells, following gel chromatography of serum-free conditioned medium on a Bio-Gel P-100 column in 1 M acetic acid. Increasing aliquots (□ > ● > ○) were lyophilized and tested for growth stimulatory activity of quiescent MCF-7 cells, as outlined in *Protocol 3*. Data are expressed relative to DNA content of cells stimulated by 9% FBS. The column was calibrated by the following marker proteins: transferrin (80 kd; fraction 26), carbonic anhydrase (29 kd; fraction 37), RNAse (13.7 kd; fraction 60), and insulin (6 kd, fraction 90). For more details see (12).

Individual column fractions were lyophilized, resuspended in a neutral buffer, and tested for growth-stimulating activity on quiescent MCF-7 cells. IGF activity elutes in three peaks, corresponding to a 6 kd low molecular weight form (fractions 60–100), a higher molecular weight (> 10 kd) precursor form (fractions 40–60), and an activity co-eluting with the proteins in the void volume of the column. This latter fraction may represent a large molecular weight precursor form, but may also result from non-specific column elution. Moreover, P19 EPI-7 cells also produce IGF-binding proteins which elute on this column in the fractions 25–35 (16). Since these binding proteins may re-associate with the co-eluting IGFs after pH neutralization, the activity observed in the high molecular weight fractions is most likely an underestimate of the amount of IGF present.

On the basis of the MCF-7 bio-assay alone, no estimate of the amount of IGF can be made, unless it is known which member of the IGF family is present. Since P19 EPI-7 cells only express the IGF-II gene, it can be assumed that the present activity mainly results from this growth factor. From a dose-response curve of the combined fractions 60–100 of this column in the MCF-7 assay, and the dose-response curve obtained for pure IGF-II, it can now be calculated that P19 EPI-7 cells secrete approximately 0.5 mg low molecular weight IGF-II per litre of conditioned medium.

5. Application of PCR technology for detection of growth factor gene expression

5.1 Introduction

As an alternative to bio-assays, the expression of known growth factors can be analysed at the mRNA level. Although the presence of mRNA for a growth factor does not necessarily imply that this factor will be produced as a bio-active polypeptide, this approach is more sensitive and specific in comparison with most bio-assays and provides important information in a very short time.

Methods for determining the presence of specific RNAs are mostly based on hybridization with a suitable complementary probe sequence. Usually, the RNA is first immobilized on a membrane (either directly, as in dot- or slot-blotting, or only after separation of total cellular RNA, or a poly(A) RNA fraction derived thereof, in denaturing agarose gels, as in northern blotting), and subsequently hybridized with an appropriate radioactivity labelled probe of sufficient length. Dot-blot hybridization has the advantage of being very fast and easily quantifiable, but requires strict control of hybridization and washing conditions in order to avoid non-specific hybridization signals. Northern blotting is more time consuming, but has the bonus of yielding significant information on the length and complexity of the detected RNA species. However, hybridization assays generally require relatively high amounts of high quality RNA (2–5 μg of poly(A) RNA, or 20–30 μg of total RNA), and the availability of a suitable probe.

Alternatively, the presence of specific RNAs may be shown using PCR technology. The RNA is first transcribed into cDNA, using either a specific antisense primer, random hexamers, or oligo(dT) as primers for the reverse transcription. The resulting (single stranded) cDNA is subsequently amplified in a PCR reaction containing target sequence-specific sense, (i.e. of the same polarity as the mRNA) and antisense primers. PCR products can then be analysed in agarose gels, and visualized after electrophoresis either directly by ethidium bromide staining, or by blotting followed by hybridization with a suitable probe (Southern blotting). Especially in those cases where the amount of RNA is limited, the speed and sensitivity of PCR make it the method of choice for detecting low levels of RNA, provided however the sequence of the target RNA is known. The major disadvantage of this technique lies in the difficulty of making it quantitative. This is due to the inherent dependency of PCR efficiency on a number of reaction conditions (primer sequence, target sequence, buffer composition and salt concentration, primer annealing temperature, etc.). Therefore, reliable PCR quantitations can only be performed by inclusion at different concentrations of an internal control template that contains the same primer hybridization sites as the target sequence (21). Since both templates are subject to the same

reaction conditions, they are assumed to be amplified with equal efficiency. The amount of amplified target product may then be compared with the amount of amplified reference product (of which the initial concentration is known), allowing a precise estimate of the original target concentration. However, if only the presence or absence of a certain RNA is to be determined, and if exact quantification is not a pre-requisite, these internal controls may be omitted. Important parameters for PCR have recently been reviewed (22, 23), and are briefly discussed in the following section.

5.2 Important parameters for the PCR approach

5.2.1 Preparation of RNA from cultured cells

Because of the sensitivity and specificity of reverse transcription RT-PCR, preparation of poly(A) RNA is usually not necessary. In many cases, even the quality of the RNA does not appear to be critical, provided the PCR primers are located relatively close to one another in the known sequence, (e.g. less than 500 nucleotides). Therefore, most RNA preparation methods yield suitable material for PCR. We routinely use the acid phenol guanidinium isothiocyanate method (24), because it is fast and yields good quality RNA from a variety of sources (see *Protocol 4*).

Protocol 4. RNA preparation

Important: all solutions and materials must be RNase-free, follow standard procedures for working RNase-free throughout the experiment (for more information on this, see reference 30).
Solutions needed:

- denaturing solution: 4 M guanidinium isothiocyanate, 25 mM Na citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol (β-EtSH); this solution is stable for three months at 4°C in the absence of β-EtSH, and for one month if β-EtSH has been added
- phenol, equilibrated with water
- chloroform/isoamyl alcohol (50/1; v/v)
- 2 M Na acetate, pH 4.0
- isopropanol

1. Collect cells by low speed centrifugation and store as a pellet at −70°C (use only tubes that are phenol and chloroform resistant). For PCR purposes, washing of the cells before storage is not essential.
2. Add 1 ml denaturing solution per 10^7 cells (add the solution to the *frozen* pellet as this prevents, or significantly reduces, RNA degradation upon thawing).
3. Vortex vigorously to effectively lyse the cells (this may take some time because the solution becomes viscous).

Protocol 4. *Continued*

4. Add 0.1 ml 2 M Na acetate pH 4.0, and mix by vortexing.
5. Add 1 ml water-equilibrated phenol, and mix by vortexing.
6. Add 0.2 ml chloroform/isoamyl alcohol, vortex well, and keep the mixture on ice for 15 min.
7. Transfer the solution to microcentrifuge tubes and centrifuge for 20 min in a microcentrifuge (4°C, maximum speed).
8. Transfer aqueous (top) phase to a new tube (taking care to avoid the interphase), and precipitate the RNA by addition of one volume of isopropanol. Keep this RNA precipitation at −20°C for at least 1 h. Usually the RNA forms a cloudy precipitate; if white 'threads' are formed, the RNA is most likely contaminated with genomic DNA.
9. Spin down the RNA in a microcentrifuge for 10–20 min (4°C, maximum speed).
10. Remove the supernatant carefully, dry the pellet, and re-dissolve the pellet in 0.3 ml denaturing solution (as the pellet often only dissolves with difficulty, this can be done in a shaking heating block, for example Eppendorf type, at 60°C).
11. Precipitate the RNA again by addition of 0.3 ml isopropanol (see steps **8–10**).
12. Wash the pellet carefully with 80% ethanol. Do not allow the pellet to dry completely since this will make it very difficult to dissolve the RNA.
13. Dissolve the pelleted RNA in RNase-free water, or in a buffer that is compatible with subsequent procedures. Start cDNA synthesis (*Protocol 5*), or store RNA at −20°C after addition of 200 mM K acetate pH 4.5, and 2.5 volumes of ethanol (incubate at 60°C if necessary). For keratinocytes, a yield of approximately 100 μg of RNA per 10^7 cells can be expected.

5.2.2 Reverse transcription

For efficient transcription of mRNA into cDNA (both in terms of length and amount of cDNA) (see *Protocol 5*), the method of denaturation of the RNA and the choice of the primers used in the reaction seem to be of utmost importance. Although heat denaturation of the RNA followed by quick cooling on ice often works well, we have repeatedly encountered difficulties for RT-PCR of certain RNAs when using this method. Denaturation by methyl mercury hydroxide results in significant improvement in these cases. For the choice of primers used for reverse transcription, different options are available. Oligo(dT) primers work well, but are less efficient if the target sequence for PCR is located near the 5′ end of a long mRNA. Indeed, in

those cases the cDNA has to be nearly full-length to make PCR possible, which is difficult if the RNA is of poor quality, or if it contains regions where the reverse transcriptase tends to stop or pause. This problem can be circumvented by using a specific reverse transcription primer which is located closer to the 3′ end of the mRNA than the antisense PCR primer. However, this requires a separate reverse transcription primer and a separate cDNA preparation for each target sequence to be determined, adding significantly to the cost and workload of the analysis. Theoretically, the same antisense primer can be used both for RT and PCR. However, we find this often results in the generation of non-specific PCR products, presumably because of mispriming during reverse transcription (where annealing conditions are usually much less stringent).

Altogether, we have obtained the best results using random hexamer primers, both in terms of yield of and specificity of amplified DNA. This offers the additional advantage of allowing the use of multiple PCR primer sets on the same preparation of cDNA. One potential disadvantage, however, is that the amplification of very long fragments (e.g. longer than 2000 nucleotides) may be less efficient, since the length of randomly primed cDNA is on the average shorter than that of oligo(dT) primed cDNA.

Protocol 5. cDNA preparation

1. Add to a microcentrifuge tube and mix well:
   - total RNA 1 μg
   - 100 mM methyl mercury hydroxide 0.5 μl
   - RNase-free water to 5 μl

   Caution! Methyl mercury hydroxide is volatile and extremely toxic: handle it only in a fume hood! Wear gloves!
2. Incubate at 24°C for 10 min.
3. Put the mixture on ice and inactivate the methyl mercury hydroxide by addition of 2.5 μl of 140 mM β-mercaptoethanol.
4. Incubate on ice for 5 min and add:
   - placental ribonuclease inhibitor (Amersham) 15 U
   - 5 × Superscript buffer (BRL) 3 μl
   - 100 mM DTT 2 μl
   - random hexamer primers, 0.5 μg/ml (Pharmacia) 1 μl
   - water to 15 μl
5. Incubate at 24°C for 5 min (primer annealing) and add:
   - dNTP mixture (10 mM of each dNTP) 2 μl
   - 5 × Superscript buffer 1 μl

Protocol 5. *Continued*

   - water 1.5 μl
   - Superscript reverse transcriptase, 200 U/ml (BRL) 0.5 μl
6. Incubate at 37°C for 90 min. Proceed to PCR (*Protocol 6*), or store at −70°C until further use.

5.2.3 PCR

i. *Primer selection*

For selection of PCR primers, a number of parameters have to be taken into account. Care should be taken to avoid complementarity between the 3′ ends of the primer oligonucleotides since this may result in excessive primer dimer formation and, consequently, in low PCR product yields. Also, it is advisable to choose primers with a similar melting temperature, since this guarantees that a single annealing temperature which is optimal for both primers can be chosen. In general, primers with melting temperatures between 55 and 65°C have proven most satisfactory for our purposes. The G + C content of both primers should preferably be between 40 and 60%, with A/T and G/C residues spaced evenly along the primer sequence. In most cases, primers are chosen so that the resulting PCR product contains a 'diagnostic' restriction site. This site enables easy confirmation of the identity of the PCR product. Selection of PCR primers is facilitated by using dedicated computer programmes, such as the one of Rychlyk *et al.* (25). This programme calculates the melting temperatures of the selected primers, and checks for potential hairpin structures and primer dimer formation. In addition, an estimation of the optimal annealing temperature is made.

ii. *PCR*

Usually, only a small quantity of cDNA (equivalent to the amount obtained by reverse transcription of approximately 50 ng of total RNA) is used for PCR (see *Protocol 6*). In cases where the target sequence is abundant, (e.g. for positive controls such as β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs), this cDNA has to be additionally diluted 10 to 1000-fold. Primers used for the first time are tested at three different annealing temperatures: the temperature calculated by the computer programme (see *i.*), and temperatures 5°C under or above this calculated temperature, respectively. In order to increase the specificity and to decrease the formation of primer dimers, it is advisable to use a so-called 'hot start' procedure. This means the reaction mixture is heated in the thermocycler for three minutes to 95°C in the absence of one key component (usually Taq polymerase). The reaction mixture is subsequently cooled to the annealing temperature and the reaction started by addition of the missing component. This protocol precludes primer annealing and extension at temperatures lower than the optimal anneal-

ing temperature, lowering the probability of mispriming. The number of cycles has to be optimized for each primer set, and usually varies between 30 and 50 cycles, depending on the abundance of the target sequence. Visualization of PCR products is done by analysing a sample of the reaction mixture by agarose gel electrophoresis. For more sensitive detection, PCR products may also be visualized by Southern blotting, using a suitable probe for hybridization.

Protocol 6. PCR (50 μl reaction)

PCR reactions are carried out in siliconized 500 μl microcentrifuge tubes *without evaporation vents*. Extreme care must be taken to avoid cross-contamination of samples, and contamination of pre-amplification reactions with post-amplification material. Because of the sensitivity of the technique, this will inevitably lead to false positives. To check on this, always introduce appropriate controls, (e.g. reactions without cDNA template). It is also advisable to dedicate separate laboratory space for pre-PCR, PCR, and post-PCR manipulations.

1. Add together in the tube[a]:
   - cDNA (see *Protocol 5*) 1 μl
   - PCR primers (20 pmol/μl) 1 μl each
   - 5 mM dNTP mix 2 μl
   - 10 × PCR buffer (Stratagene) 5 μl
   - Taq polymerase (Stratagene) 2 U
   - water to 50 μl
2. Overlay reaction with paraffin oil or mineral oil to avoid evaporation.
3. Carry out PCR reaction in a thermal cycler using the following cycling scheme:
   (a) denaturation (95°C) 3 min
   (b) annealing (T_a)[b] 1 min
   (c) extension (72°C) 2 min
   (d) denaturation (95°C) 20 sec
   repeat steps (b) to (d) *n* times[c]
   (e) annealing (T_a) 1 min
   (f) extension (72°C) 10 min
4. Analyse PCR products by agarose gel electrophoresis followed by ethidium bromide staining.

[a] For performing a 'hot start' reaction, Taq polymerase is added only after the initial 3 min denaturation step (annealing time in the first step can be extended for this purpose).
[b] Optimal annealing temperature (T_a) should be determined for each primer set (see text).
[c] Number of cycles (*n*) is dependent on the target abundance and should also be optimized.

6. Example: RT-PCR on human keratinocyte RNA

Cultured epidermal cells are used by several clinical centres to improve healing of several types of wounds, such as deep second and third degree burn wounds, chronic ulcers, and skin donor sites (26–28). It is widely accepted that this wound healing-promotion effect can, at least partly, be attributed to polypeptide growth factors produced by keratinocytes. As part of our studies into keratinocyte graft mediated wound healing, we routinely evaluate by PCR the presence of the mRNAs of several known growth factors in cell sheets of primary human keratinocytes.

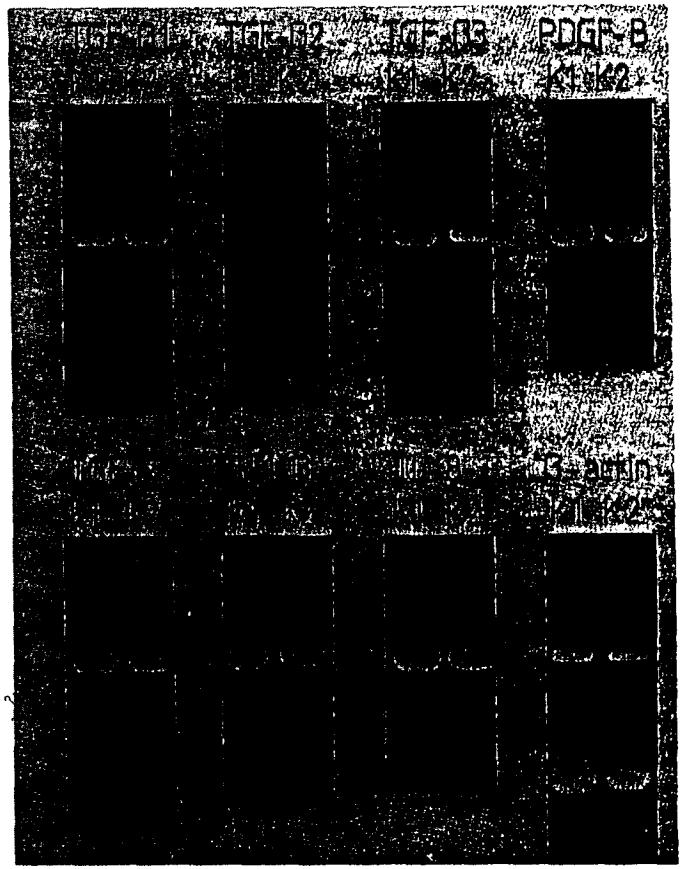

Figure 5. PCR analysis on human keratinocyte cDNA using different primer sets. cDNA was prepared from RNA from two different human keratinocyte preparations (K1 and K2). PCR was performed as described in *Protocol 6*, using primers for the indicated polypeptides (see also *Table 1*). All PCR reactions were performed using 50 cycles. For the PCR of β-actin, the cDNA was diluted 100-fold prior to the reaction. 20 μl of each reaction was run in a 2% agarose gel and PCR products were visualized by ethidium bromide staining after electrophoresis.

Human keratinocytes derived from skin donor sites can be grown on growth-inhibited 3T3 'feeder' layers (either irradiated or mitomycin C treated) as described earlier (29). Near-confluent keratinocytes (passage 2) are collected by dispase treatment and used for preparation of RNA. In the experiments which are briefly illustrated here, we have tested the cells for the following cytokine mRNA sequences: TGF-α, TGF-β1, TGF-β2, TGF-β3, interleukin(IL) 1β, IL-8, and platelet-derived growth factor (PDGF)-B (for primer sequences, see *Table 1*). As a control on the quality of the RNA preparation and on the efficiency of the reverse transcription, primers for β-actin were included. The number of PCR cycles and the amount of starting cDNA were adjusted for each primer set in order to ensure the plateau of reaction limiting conditions was not reached.

As can be seen in *Figure 5*, mRNA for nearly all of the cytokines mentioned above was present in approximately equal amounts in the two keratinocyte preparations (primary cell cultures from two different patients) tested. Only TGF-β2 mRNA was present in much lower amounts in one preparation and nearly absent in the other. Although these data are not quantitative, and do not necessarily imply that the investigated growth factors are produced in a bio-active form by keratinocytes, they nevertheless support the view that these cells may play an important role in the triggering or the maintenance of wound healing through the synthesis and secretion of different cytokines.

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Table 1. PCR primers for detection of mRNA of some human polypeptides by PCR

Target sequence	Oligonucleotide sequence	Primer[a]	Length of PCR product (bp)	T_a (°C)[b]
hTGF-α	5′-GCCCGCCCGTAAAATGGTCCCTC-3′	s	527	65
	5′-GTCCACCTGGCCAACTCCTCCTCTGGG-3′	as		
hTGF-β1	5′-CGCCTTAGCGCCCACTGCTCCTGTGT-3′	s	533	65
	5′-GGGGCGGGACCTCAGCTGCACTTG-3′	as		
hTGF-β2	5′-GCTCTGTGGGTACCTTGATGCCATCC-3′	s	573	60
	5′-TTCTTCCGCCGGTTGGTCTGTTGTG-3′	as		
hTGF-β3	5′-ACTGCCGAGTGGCTGTCCTTTGATG-3′	s	538	60
	5′-AGGCAGATGCTTCAGGGTTCAGAGTG-3′	as		
hIL-1β	5′-ATGCTGGTTCCCTGCCCACAGACCTTCC-3′	s	550	60
	5′-TTATATCCTGGCCGCCTTTGGTCCTCC-3′	as		
hIL-8	5′-GGACAAGAGCCAGGAAGAAACCACC-3′	s	516	55
	5′-GGCAACCTACAACAGACCCACAC-3′	as		
hPDGF-B	5′-TGCACGGAGACCCCGGAGAGGAAGATGG-3′	s	497	65
	5′-GCACCGTCCGAATGGTCACCCGAGTTTG-3′	as		
hβ-actin	5′-GTACGTTGCTATCCAGGCTGTGCTATCC-3′	s	504	60
	5′-GACAGCACTGTGTTGGCGTACAGGTC-3′	as		

[a] s = sense primer (has mRNA polarity), as = antisense primer
[b] T_a refers to the annealing temperature used for PCR (in °C)

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3

Induction of DNA synthesis and cell division in quiescent cells in response to small peptide growth factors

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1. Introduction

Among the most frequently used model systems for the study of cell growth control are a number of mouse embryo-derived fibroblastic cell lines, termed 3T3 cells. The terminology reflects the original experiments designed to derive and maintain cells in a non-transformed, non-tumourigenic state, (i.e. cells which depended on the presence of serum for their growth, which did not overgrow each other, and which did not cause tumours when injected into recipient animals). It was found that passaging the cells every three days at 3×10^5 cells/5 cm dish maintained the cells in optimum condition by those criteria (1). Three lines of these cells are in common use, namely Swiss, Balb, and NIH 3T3 cells; the names reflect the different strains of mice from which the cells were derived, using similar methods. Although the cells are commonly described as fibroblasts, the embryonic cell of origin is not established.

In addition to those molecules originally discovered and named for their growth factor activity (epidermal growth factor (EGF), platelet-derived growth factor (PDGF), etc.) a variety of smaller peptide mitogens (typically less than twenty amino acids) also promote DNA synthesis and cell division in Swiss 3T3 cells (*Table 1*). These peptides are often hormones that have defined roles in regulating cellular functions other than proliferation. Several points may be made about cell stimulation by these peptides.

(a) Their mitogenic activity is not limited to Swiss 3T3 cells. For example, the regulatory peptide bombesin stimulates DNA synthesis in cell lines derived from small cell lung cancers, and anti-bombesin antibodies have been reported to suppress the growth of these cells *in vivo* (2), supporting the view that autocrine stimulation by bombesin related peptides contributes to their deregulated proliferation.

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Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays

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A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multiwell scanning spectrophotometer (ELISA reader) and show a high degree of precision. No washing steps are used in the assay. The main advantages of the colorimetric assay are its rapidity and precision, and the lack of any radioisotope. We have used the assay to measure proliferative lymphokines, mitogen stimulations and complement-mediated lysis.

Key words: *lymphokine assays – proliferation assays – colorimetric assay – tetrazolium – TCGF*

Introduction

Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by several methods, e.g., counting cells that include/exclude a dye, measuring released ^{51}Cr-labeled protein after cell lysis, and measuring incorporation of radioactive nucleotides ([^{3}H]thymidine or [^{125}I]iododeoxyuridine) during cell proliferation. The radioactive method can be partially automated and can handle moderately large numbers of samples, but even with these methods, it is difficult to process thousands of assay points per day. In our current research we assay many samples of various lymphokines that induce cell proliferation, and so we required a rapid and quantitative assay capable of handling large numbers of samples.

Viable cells could be measured by using any of several staining methods, but we wished to avoid any washing steps that would increase processing time and sample variation. Multiwell scanning spectrophotometers (ELISA readers) can measure large numbers of samples with a high degree of precision, and so we investigated the possibility of using a color reaction as a measure of viable cell number. Ideally, a

colorimetric assay for living cells should utilize a colorless substrate that is modified to a colored product by any living cell, but not by dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes (Slater et al., 1963). The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells.

We have developed a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), that measures only living cells and can be read on a scanning multiwell spectrophotometer (ELISA reader). This assay is versatile and quantitative, and we consider it a significant advance over traditional techniques for several commonly used proliferation and cytotoxicity assays.

Materials and Methods

Cell lines

The EL_4G^- mouse lymphoma cell line was obtained from G. Carlson, and subclone EL_4.3 was selected for growth in 6-thioguanine. Another subline of EL_4, designated EL_4E2, was obtained from V. Paetkau. The EL_4E2 subline produces large quantities of interleukin 2 when stimulated with phorbol myristate acetate (Farrar et al., 1980). A continuous line of mouse T cells, A70 13/13, was derived in the author's laboratory at the University of Alberta. All cells were grown in RPMI 1640 supplemented with 50 μM 2-mercaptoethanol and 5–10% fetal bovine serum, in a 6% CO_2 atmosphere.

Colorimetric MTT (tetrazolium) assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, stock MTT solution (10 μl per 100 μl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Dynatech MR580 Microelisa reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99 (or 1.00 if the samples were strongly colored). Plates were normally read within 1 h of adding the isopropanol.

Interleukin 2 assay

Interleukin 2 (IL2) was derived from phorbol-myristate-acetate stimulated EL_4E2 cells (Farrar et al., 1980). The IL2-dependent T cell line A70 13/13 was used as an indicator cell. Doubling dilutions of IL2 were prepared in 96-well trays using growth medium as diluent. T cells (2000 per well) were then added, with a final volume of 0.1 ml per well. At 48 h, proliferation was measured by the MTT colorimetric assay.

Mitogen-induced proliferation of spleen cells

BALB/c mouse spleen cells were stimulated in 0.1 ml at 10^6 cells/well with varying concentrations of *Salmonella typhosa* lipopolysaccharide (LPS; Sigma) or concanavalin A (Con A; Calbiochem) and assayed at 3 days for proliferation using both colorimetric MTT and [^{3}H]thymidine incorporation assays. For the radioactive assay, 0.001 mCi [^{3}H]thymidine was added to each well, and after 4 h at 37°C the cells were harvested using a PHD cell harvester (Cambridge Instruments, Cambridge, MA).

Computer processing

Readings from the Dynatech MR580 Microelisa reader were transferred directly to an Apple II computer, using a program that saved the results to a diskette and printed the OD values in a 96-well format that matched the original plate. Additional programs were written to process the data stored on diskettes. We now have programs to plot results, calculate and plot means and standard deviations, identify wells above a chosen threshold, and calculate units of growth factor. These programs are available on request.

Results

In preliminary experiments, we tested several tetrazolium salts by incubation with cells for several hours. The most promising reagent was MTT, a pale yellow substrate that produced a dark blue formazan product when incubated with live cells. The MTT formazan reaction product was only partially soluble in the medium, and so an alcohol was used to dissolve the formazan and produce a homogeneous solution suitable for measurement of optical density. Initially, ethanol was used for this purpose, but some precipitation of serum proteins occassionally occurred in the acid-alcohol mixture. Several other organic solvents were tested, and isopropanol was found to be the most suitable solvent. Normal tissue culture medium has a variable color due to pH changes and the red form of phenol red interfered at the wavelength most suitable for blue MTT formazan measurement. To minimize this interference, we converted the phenol red to the fully acidic, yellow form at the end of the assay.

Our final procedure was to add 0.01 ml MTT (5 mg/ml in phosphate-buffered saline) to 0.1 ml cells in growth medium. After 4 h at 37°C for MTT cleavage, the formazan product was solubilized by the addition of 0.1 ml 0.04 N HCl in isopropanol. Optical density was measured on a Dynatech MR 580 plate reader, using a reference wavelength of 630 nm and a test wavelength of 570 nm.

EL_4.3 lymphoma cells were used to test the relationship between cell number and the amount of MTT formazan generated. The results in Fig. 1 show that the absorbance is directly proportional to the number of cells. This linearity extends over almost the entire range tested, from 50,000 to 200 cells/well. In addition, these results indicate that the assay is capable of detecting very small numbers of living cells (e.g., 200). The actual cells do not absorb significantly, even at a concentration of 1×10^6 cells/ml.

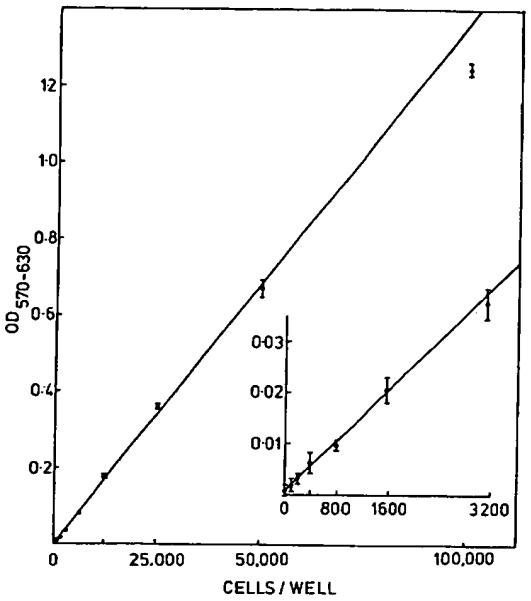

Fig. 1. Linearity of the MTT assay. EL_4.3 cells were plated out in doubling dilutions in 0.1 ml growth medium (RPMI 1640 + 10% fetal bovine serum) in 96-well flat-bottomed trays (Falcon), starting at 10^5 cells/well. MTT (0.01 ml of 5 mg/ml stock) was added immediately to all wells, and the plates were incubated at 37°C for 3 h, developed and measured. Each point shows the mean and standard deviation of 4 replicates. The straight line plotted is the best fit line calculated using all points from 100 to 50,000 cells/well.

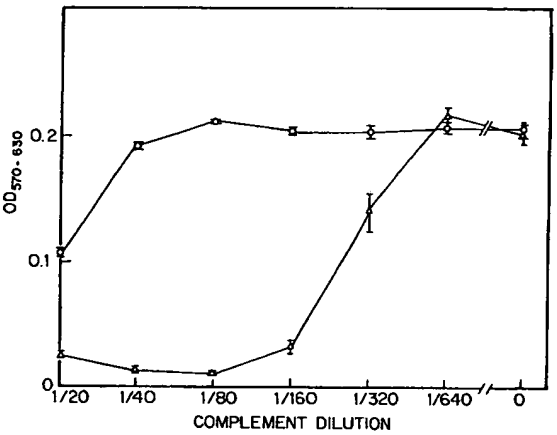

Fig. 2. Living cells are required for MTT cleavage. EL_4.3 cells were treated with anti-Thy1.2 (Cedarlane), and then treated cells and untreated controls were added to rabbit complement dilutions in a 96-well tray. After 30 min incubation at 37°C, MTT was added to all wells, and after another 4 h the plates were developed and read. Each point shows the mean and standard deviation of 4 replicates. △, anti-Thy1.2-treated; O, untreated.

In many assays, dead cells will be present, and so it was important to determine if recently killed cells were positive or negative in the assay. Fig. 2 shows that only live cells actively cleave MTT, while dead cells are almost completely negative even immediately after complement-mediated lysis. These results suggested that living cells with active mitochondria are required to generate a strong signal, and raised the possibility that the amount of formazan generated per cell would depend on the level of energy metabolism in the cell. To test this, we measured formazan generation by metabolically inactive cells (red blood cells), resting cells (spleen cells) and activated cells (concanavalin A-stimulated lymphocytes). Fig. 3a shows that neither chicken nor sheep red blood cells cleave MTT to a significant extent, and neither red cell type interferes significantly in the assay, up to concentrations of 2×10^6 cells/ml. Fig. 3b shows that Con A-activated lymphocytes produce approximately 10 times as much formazan per cell as their normal counterparts.

A continuous line of interleukin 2 (IL2)-dependent T cells (A70 13/13), previously established in the author's laboratory at the University of Alberta, was used as

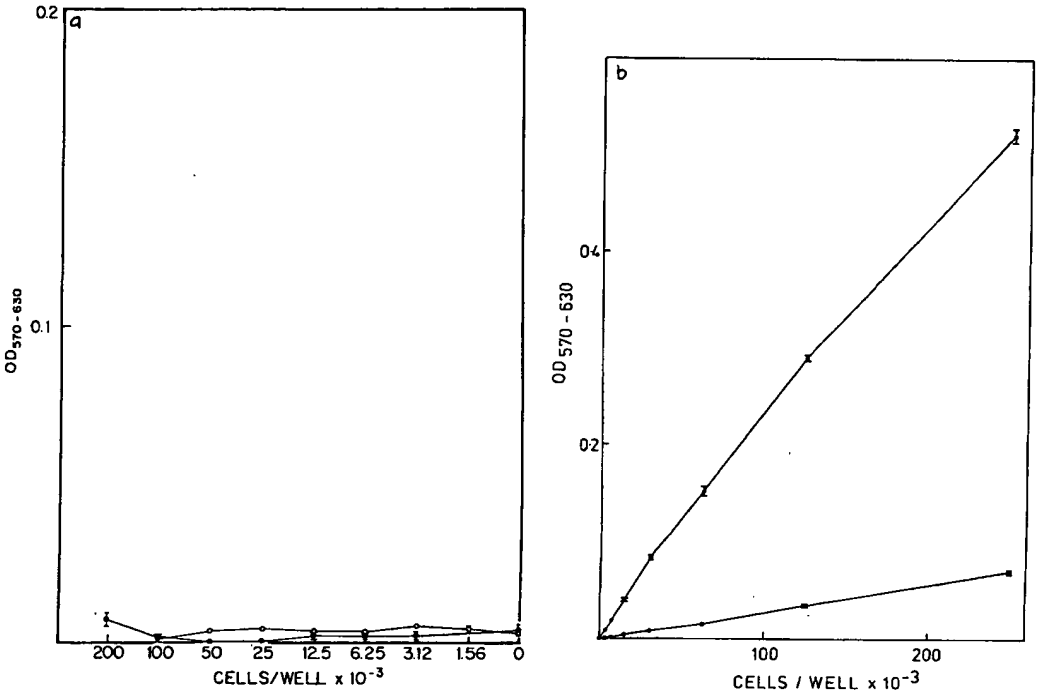

Fig. 3. MTT cleavage by erythrocytes and normal and activated lymphocytes. a: chicken and sheep erythrocytes were incubated in 0.1 ml medium with MTT for 3 h at 37°C. The plates were then developed and read. Means and standard deviations of 3 replicates per point are shown. ●, chicken erythrocytes; ○, sheep erythrocytes. b: mouse spleen cells were stimulated with 2 μg/ml concanavalin A for 48 h, and then the Con A-activated lymphocytes and normal lymphocytes were plated in doubling dilutions in 96-well flat-bottomed trays. MTT was added immediately, and after 2 h, the plates were developed and read. The means and standard deviations of 3 replicates per point are shown: ●, Con A-activated lymphocytes; ○, normal lymphocytes.

the target cell for an IL2 assay. This assay was used to test 4 parameters of the colorimetric reaction – the length of exposure of cells to IL2, the duration of MTT treatment, the concentration of MTT used, and the number of test cells added to the assay.

Fig. 4 shows the effect of varying the time of incubation with MTT. The signal increased almost linearly from 1/2 to 2 h, but increased at a lesser rate from 2 to 4 h. In similar experiments, the concentration of MTT and cell number were optimized for the cell lines used in our studies (results not shown). The formazan generated was approximately proportional to the MTT concentration at low concentrations, and reached a plateau at about 0.45 mg/ml MTT. The formazan generated was also proportional to the number of cells at high IL2 concentrations, but the amount of factor required to produce 50% stimulation was increased at higher target cell concentrations. The assay could be read at 1, 2 or 3 days, but the apparent titer of the IL2 declined with increasing incubation time, probably due to depletion of the growth factor during cell growth. Our optimum values for these 4 parameters may need modifying for other assays, but in general, we have found that widely differing cell lines require only minimal changes.

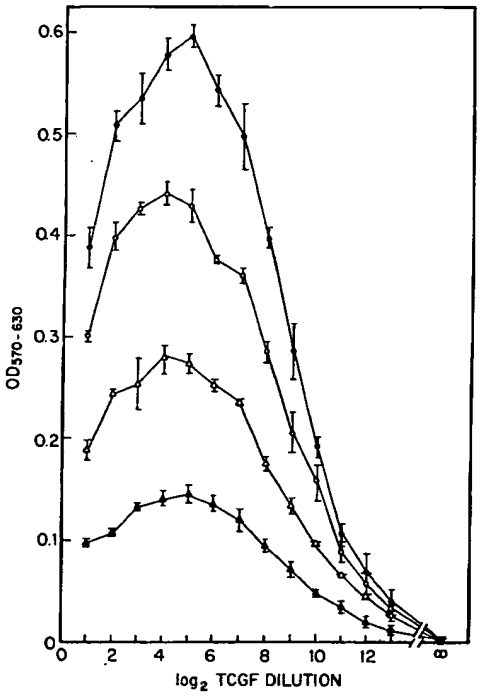

Fig. 4. Duration of MTT incubation. Interleukin 2 dilutions were assayed on A70 13/13 T cells. MTT was added at 44, 46, 47 and 47½ h, to sets of 3 rows each, and at 48 h, all wells were developed and read. Optical density readings were measured relative to control wells containing medium, cells and MTT but no growth factor. The means and standard deviations of 3 replicates per point are shown. ▲, 1/2 h; △, 1 h; ○, 2 h; ●, 4 h.

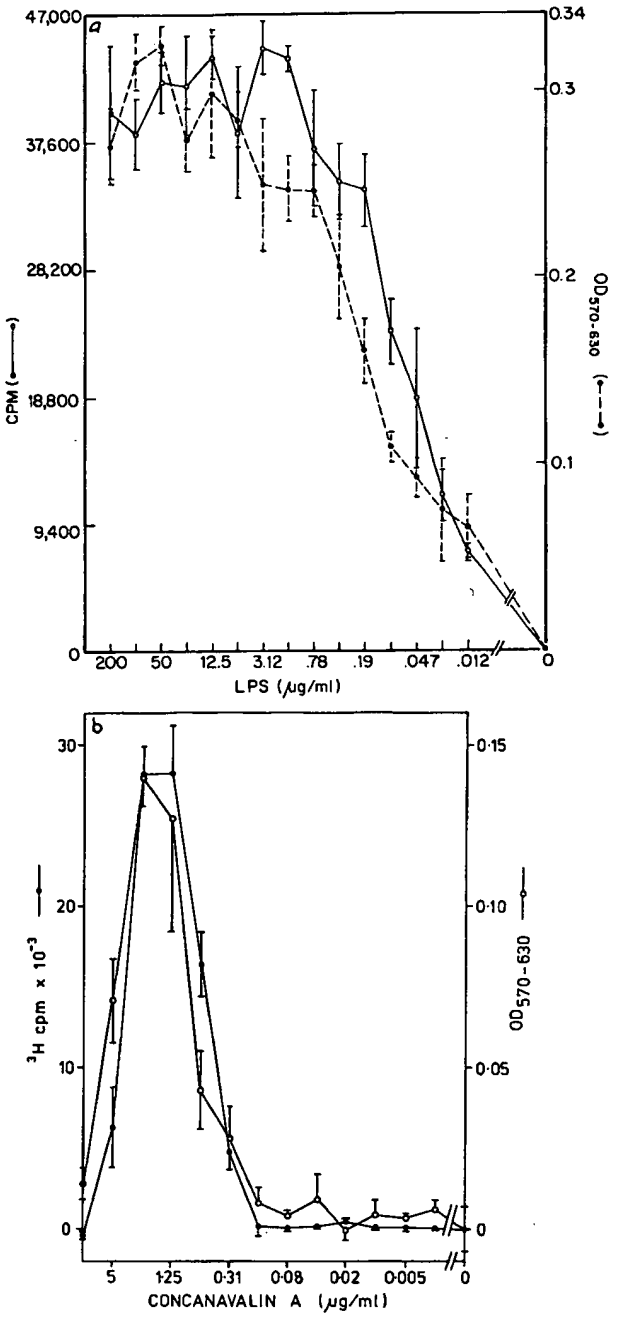

Fig. 5. Mitogen-induced proliferation of spleen cells. Spleen cells were stimulated for 3 days with varying concentrations of LPS and Con A and proliferation was measured using both colorimetric and radioactive assays. Results are shown as the means and standard deviations of 4 replicates per point. Background values, obtained from wells with cells but no mitogen, were subtracted from all points. a: LPS stimulation. b: Con A stimulation.

The results of the colorimetric assay with cloned cell lines were very encouraging, and so we explored the utility of the assay in more complex systems: the lymphocyte proliferative responses to the mitogens Con A and LPS. Since many cell types are present in the cell populations normally used for such proliferations, it was conceivable that certain cell types would generate abnormally large or small signals. Accordingly, we compared the colorimetric assay to a [^{3}H]thymidine incorporation assay for both Con A and LPS responses of normal mouse spleen cells.

Stimulation with both mitogens was measured effectively by both assays (Fig. 5). LPS stimulated cells over an extended concentration range, whereas the titration curve for Con A showed a narrow optimum, with little or no proliferation at high or low concentrations. The colorimetric and radioactive assays showed excellent agreement for Con A stimulations, and showed a small difference between the endpoint of LPS stimulations.

Activated macrophages produce more formazan product from nitroblue tetrazolium than do non-activated macrophages (Baehner et al., 1976), and so we measured MTT formazan production after LPS activation of a macrophage-like cell line, P388D1 (Lachman et al., 1977). No increase in MTT formazan production was seen after stimulation with a wide range of LPS concentrations, and P388D1 cells did not produce an unusual amount of MTT formazan (results not shown).

Discussion

The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells that we have tested, but not by dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population. Activated cells produce more formazan than resting cells, which could allow the measurement of activation even in the absence of proliferation. These properties are all consistent with the cleavage of MTT only by active mitochondria.

The main advantage of the colorimetric assay is the speed with which samples can be processed. The substrate does not interfere with measurement of the product, and we have found conditions in which components of the medium do not interfere. This allows the assay to be read with no removal or washing steps, which increases the speed of the assay and helps to minimize variability between samples. The final stages of the assay (adding the MTT, reading the plate and printing the data) take much less time than setting up the assay (mixing cells and growth factor dilutions). The assay can be read a few minutes after the addition of acid-isopropanol, and the color is stable for a few hours at room temperature. The results are also apparent visually, which is very useful if rapid qualitative results are required.

The colorimetric assay measures the number and activity of living cells at the end of the assay, whereas [^{3}H]thymidine incorporation measures the number of cells synthesizing DNA during the last few hours of the assay. So the colorimetric assay correlates well with visual examination of the cells at the end of the assay (Kappler

et al., 1981) but these 2 assays can potentially differ from radioactive nucleotide incorporation methods. This should be kept in mind for specific applications, e.g., distinguishing between death, survival and proliferation. In practice, we have not seen large differences between the colorimetric assay, radioisotope assay or visual inspection of the wells.

The only additional reagents used in the assay are MTT, isopropanol, and HCl. No radioisotopes are used, and no scintillation counter or gamma-counter is needed. This advantage is partially offset by the requirement for a plate reader, but the high scanning rate of typical machines (e.g., $1\frac{1}{2}$ min per 96 wells) allows a single plate reader to handle very large numbers of samples.

The colorimetric assay shares with the radioisotope assays the advantages of precise quantitation and compatibility with computer analysis programs. Since the colorimetric assay is so rapid, large amounts of data can be generated, and some form of computer processing is very desirable. We have set up programs for calculating means and standard deviations, plotting curves, and calculating units of growth factor in the original sample (using a linear interpolation to calculate the exact dilution at which stimulation is a preset value, e.g., 25% of the maximum plateau stimulation). These programs are written for an Apple II computer, and are available on request.

The reduction of MTT to a formazan product appears to be carried out by all the cell types we have examined. These include mitogen stimulated T and B cells, myeloma, T lymphoma and macrophage-like tumor cell lines, as well as various IL2-dependent T cell lines. This suggests that the colorimetric MTT assay may have very wide applicability for measuring survival and/or proliferation of various cells and can potentially be applied to any assay in which living cells must be distinguished from dead cells or a lack of cells. The results in Fig. 2 show that dead cells are unable to cleave MTT within 30 min of complement-mediated lysis. This indicates that the assay also has potential value for quantitative and rapid measurement of cell death, e.g., in HLA typing. The MTT assay may also be applicable to the assay of cytotoxic T lymphocytes, although the signal generated by the CTL population could mask the signal from the target population at high effector : target ratios.

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TISSUE CULTURE TECHNIQUES

AN INTRODUCTION

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EXHIBIT 6

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CONTENTS

2-1

Keratinocyte Growth Factor

A Fibroblast Growth Factor Family Member with Unusual Target Cell Specificity

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INTRODUCTION

Growth factors are important mediators of intercellular communication. These potent molecules are released by cells and act to influence proliferation of the same or other cell types.[1] Interest in growth factors has been heightened by evidence of their potential involvement in neoplasia. The v-*sis* transforming gene of simian sarcoma virus encodes a protein that is homologous to the B chain of platelet-derived growth factor.[2,3] Moreover, a number of oncogenes are homologues of genes encoding growth factor receptors.[4] Thus, increased understanding of growth factors and their receptor-mediated signal-transduction pathways is likely to provide insights into mechanisms of both normal and malignant cell growth.

Recognizing that most human malignancies arise in epithelial tissues[5] where cell populations are continuously turning over, we sought to identify growth factors specific for these cell types. To screen for epithelial-specific mitogens, we employed the mouse keratinocyte line BALB/MK[6] as a prototypical epithelial cell and the NIH/3T3 fibroblast[7] as its nonepithelial counterpart. Preliminary analysis of conditioned medium from a variety of sources revealed that fibroblast cell lines produced factors capable of inducing DNA synthesis in both cell types. Whereas boiling or acid treatment eliminated the activity for BALB/MK, the activity for NIH/3T3 cells remained intact. We reasoned that the fibroblast lines were secreting heat- and acid-labile mitogen(s) with an apparent epithelial cell specificity. This interpretation also was consistent with increasing evidence that mesenchymal interactions presumably mediated by diffusible substances had a major impact on epithelial cell proliferation.[8-10] Efforts to purify and characterize the putative agent(s) responsible for the

EXHIBIT 7

Growth Factor

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activity on BALB/MK resulted in the identification of keratinocyte growth factor (KGF),[11] a mitogen structurally related to the fibroblast growth factors (FGFs), but with the distinctive properties of a paracrine mediator of epithelial cell growth.[12]

Further studies with recombinant KGF defined the biochemical characteristics of its cell surface receptors, establishing the basis for its target cell specificity.[13] Using a novel expression cloning strategy, we isolated a cDNA encoding the high-affinity KGF receptor.[14] Analysis of this receptor cDNA has provided insights into its relationship to other members of the newly emerging FGF receptor family.

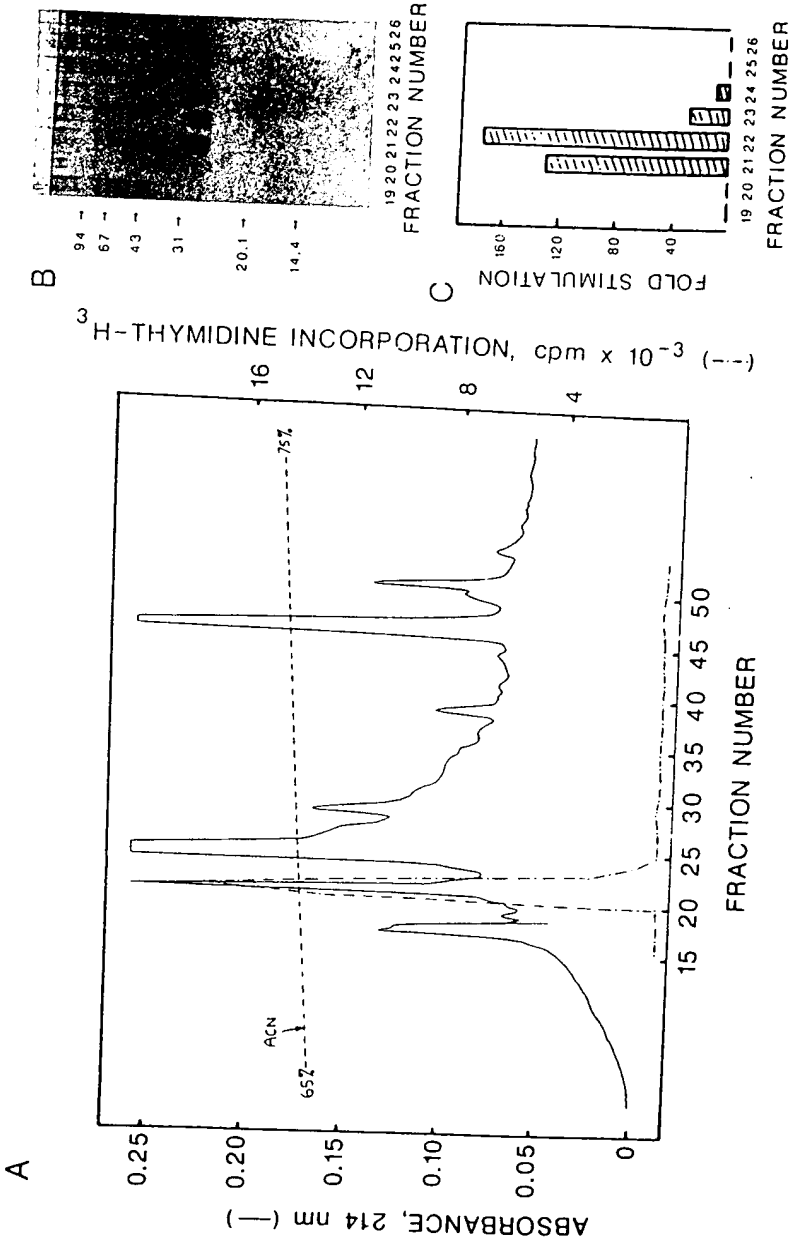
PURIFICATION OF A GROWTH FACTOR SPECIFIC FOR EPITHELIAL CELLS

M426, a human embryonic lung fibroblast line,[15] was selected as the best source for purification of the putative growth factor(s) responsible for BALB/MK mitogenic activity. Ultrafiltration provided a convenient way of reducing the volume of conditioned medium to a suitable level for subsequent chromatography. Heparin-Sepharose affinity chromatography, which has been used in the purification of other growth factors,[16–21] was the most efficient purification step. While estimates of recovered activity were uncertain at this stage because of the likely presence of multiple factors, the apparent yield was 50–70% with a corresponding enrichment of ~1000-fold. More than 90% of the BALB/MK mitogenic activity was eluted with 0.6 M NaCl and was not associated with any activity on NIH/3T3 cells.[11] Prompt concentration of 10- to 20-fold was essential for stability, which then could be maintained at −70°C for several months.

Final purification was achieved by reverse-phase high-performance liquid chromatography (RP-HPLC) (Vydac C_4 column), a preparative method suitable for amino acid sequence analysis. While the yield of activity from this step was only a few percent, the loss could be attributed to the solvents used. In other experiments, exposure to 0.1% trifluoroacetic acid/50% (vol/vol) acetronitrile for 1 hour at room temperature reduced the mitogenic activity of the preparation by 98%.[11] Nonetheless, a single peak of BALB/MK stimulatory activity was obtained (FIGURE 1A), coinciding with a distinct peak in the absorption profile. Peak fractions contained a single band with a molecular mass of 28 kDa as estimated on a silver-stained, sodium dodecyl sulfate-polyacrylamide gel (FIGURE 1B), and mitogenic activity (FIGURE 1C) correlated with the intensity of this band across the chromatographic profile. This molecular mass was in good agreement with the elution position of mitogenic activity on two different sizing columns run in solvents expected to maintain native conformation.[11] From these data, we concluded the mitogen was a single polypeptide chain with a mass of 25–30 kDa.

Its distinctive target-cell specificity was demonstrated by comparing it on a variety of cell types with other growth factors known to possess epithelial cell mitogenic activity. The factor exhibited a strong mitogenic effect on BALB/MK cells and stimulated thymidine incorporation in other epithelial cells tested (TABLE 1). In contrast, the factor had no detectable effects on fibroblasts or human saphenous vein endothelial cells,[11] or on melanocytes or PC-12 cells (unpublished observations). By comparison, TGF-α and EGF showed good activity on fibroblasts, while acidic FGF (aFGF) and basic FGF (bFGF) were mitogenic for endothelial cells as well (TABLE 1). Because of its specificity for epithelial cells and the sensitivity of keratinocytes in particular, the mitogen was designated "keratinocyte growth factor."

To establish that KGF not only would stimulate DNA synthesis but also would support sustained cell growth, we attempted to grow BALB/MK cells in a fully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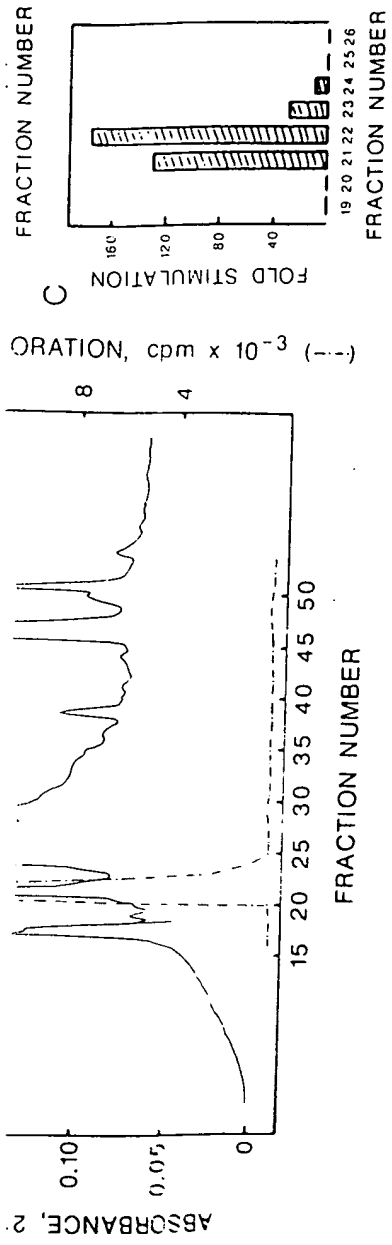


TABLE 1. Target-Cell Specificity of Growth Factors[a]

Growth Factor	Fold Stimulation of Thymidine Incorporation				
	Epithelial Cell Line			Fibroblast	Endothelial
	BALB/MK	B5/589	CCL208	NIH/3T3S	Cell Line[b]
KGF	500–1000	2–3	5–10	<1	<1
EGF	100–200	20–40	10–30	10–20	ND
TGFα	150–300	ND	ND	10–20	ND
aFGF[c]	300–500	2–3	5–10	50–70	5
bFGF	100–200	2–3	2–5	50–70	5

[a]Comparison of maximal thymidine incorporation stimulated by KGF and other growth factors in a variety of cell lines, expressed as fold stimulation over background. These data represent a summary of four different experiments. ND, not determined.
[b]Human saphenous vein cells.
[c]Maximal stimulation by aFGF required the presence of heparin (Sigma) at 20 μg/ml. aFGF and bFGF were recombinant preparations.

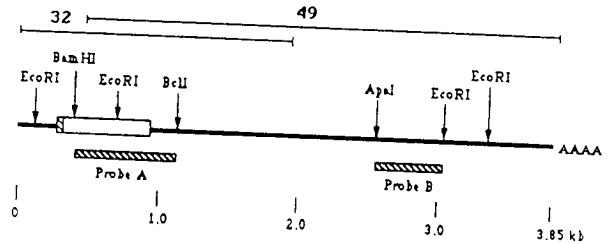
defined, serum-free medium supplemented with this growth factor. KGF served as an excellent substitute for EGF but not for insulin (or insulinlike growth factor I) in this chemically defined medium.[11] Thus, KGF acts through the major signaling pathway shared by EGF, aFGF, and bFGF for proliferation of BALB/MK cells.[22]

HUMAN KGF IS FGF RELATED WITH PROPERTIES OF A PARACRINE EFFECTOR OF EPITHELIAL CELL GROWTH

Amino acid sequence analysis of C_4-purified KGF (~150 pmol) yielded a single sequence with unambiguous assignment for cycles 2–13 as follows: Xaa-Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala-Thr-Asn-Val.[11] Oligonucleotide probes were generated on the basis of this experimentally determined amino acid sequence and then used to screen an oligo (dT)-primed cDNA library prepared from M426 human embryonic lung fibroblasts, the initial source of the factor. Of 10 plaque-purified clones analyzed, 1 (designated clone 49) had an insert of 3.5 kilobases (kb), whereas the rest had inserts ranging from 1.8 to 2.1 kb. Analysis of the smaller clones revealed several common restriction sites. Nucleotide sequencing of a representative clone (desig-

FIGURE 1. A: C_4 reverse phase high performance liquid chromatography (HPLC) of BALB/MK mitogenic activity. Active fractions eluted from the heparin-Sepharose column with 0.6 M NaCl were concentrated and loaded directly onto a Vydac C_4 column (4.6 × 250 mm) that had been equilibrated in 0.1% trifluoroacetic acid/20% acetonitrile. After the column was washed with 4 ml of equilibration buffer, the sample was eluted with a modified linear gradient of increasing percentage of acetonitrile. Fraction size was 0.2 ml, and flow rate was 0.5 ml/min. Aliquots for the assay of [^{3}H]thymidine incorporation in BALB/MK cells were promptly diluted 1:10 with 50 μg of bovine serum albumin per ml/20 mM Tris-HCl, pH 7.5, and tested at a final dilution of 1:200. B: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) analysis of selected fractions from the C_4 chromatography shown in A. Half of each fraction was dried, redissolved in SDS/2-mercaptoethanol, heat denatured, and electrophoresed in a 14% polyacrylamide gel which was subsequently silver-stained. The position of each molecular mass marker (in kDa) is indicated by an arrow. C: DNA synthesis in BALB/MK cells triggered by the fractions analyzed in B. Activity is expressed as the fold stimulation over background, which was 100 cpm.

A.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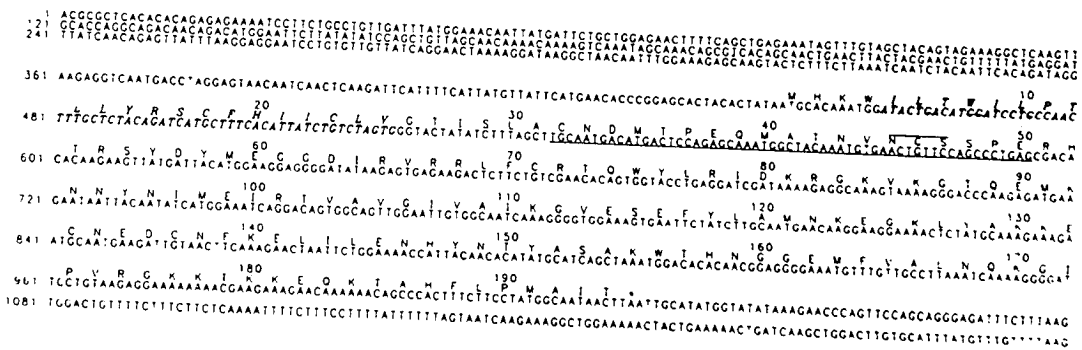

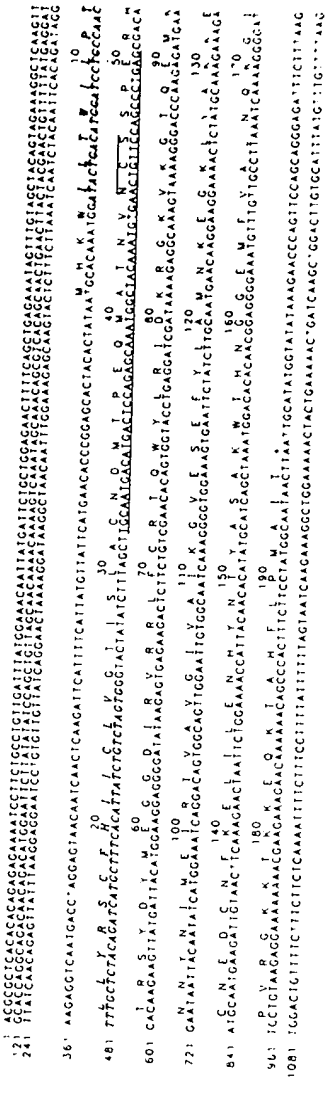

nated clone 32) along with clone 49 demonstrated that they were overlapping cDNAs (FIGURE 2A), which when aligned established a continuous 3.85-kb sequence that contained the complete KGF coding domain[12] (FIGURE 2B).

A likely ATG initiation codon was located at nucleotide position 446, establishing a 582-base-pair (bp) open reading frame, which ended at a TAA termination codon at nucleotide position 1030. This open reading frame encodes a putative 194-amino-acid polypeptide with a calculated molecular size of 22,512 daltons. A 19-amino-acid sequence, which was consistent with the experimentally determined NH_2-terminal sequence of purified human KGF, began 32 amino acids downstream of the proposed initiation codon. The predicted KGF amino acid sequence contained one potential N-linked glycosylation site (Asn-X-Ser) from residues 45 through 47.

To search for homology between KGF and any known protein, we analyzed the National Biomedical Research Foundation data base with the FASTP program of Lipman and Pearson.[23] The predicted primary structure of KGF was related to those of aFGF and bFGF, as well as *int-2*-, *hst*/KGFG, FGF-5, and FGF-6-encoded proteins. The FGFs are heparin-binding mitogens with broad target cell specificities.[24] FGF-5[25] and *hst*/KGFG[26,27] are transforming genes, originally detected by DNA-mediated gene transfer, whereas *int-2* was identified as an oncogene by proviral integration of mouse mammary tumor virus.[28,29] FGF-6 also is a transforming gene which was initially identified on the basis of homology to a human *hst*/KFGF probe.[30] Alignment of the seven proteins revealed two major regions of homology, spanning amino acids 65 to 156 and 162 to 189 in the predicted KGF sequence, which were separated by a short nonhomologous series of amino acids. In the aligned regions, KGF was 30 to 45% identical to the other six members of the FGF family.

The primary KGF translation product, like those of *hst*/KFGF, FGF-5, and FGF-6, contains a hydrophobic NH_2-terminal region. Evidence that this NH_2-terminal domain is not present in the mature KGF molecule (FIGURE 2B) indicates that it represents a signal peptide sequence.[31] Acidic and basic FGF are synthesized apparently without signal peptides.[32,33] The *int-2*-encoded protein contains an atypically short region of NH_2-terminal hydrophobic residues,[34] which apparently functions as a signal sequence.[35] The *int-2*- and FGF-5-encoded proteins also contain long COOH-terminal extensions compared to the other family members.

A probe spanning most of the KGF coding sequence (FIGURE 2A, probe A) detected a predominant 2.4-kb transcript as well as a less abundant, ~5-kb transcript

FIGURE 2. Nucleotide sequence and deduced amino acid sequence of KGF cDNA. **A:** Representation of human KGF cDNA clones. Overlapping clones 32 and 49, used in sequence determination, are shown above a diagram of the complete coding sequence as well as adjacent 5′ and 3′ untranslated regions. Untranslated regions are represented by a line; the coding sequence is boxed. The hatched region represents sequences that encode the putative signal peptide. Selected restriction sites are indicated. The derivation of two cDNA probes used for RNA blot analysis is indicated. **B:** Complementary DNA nucleotide sequence encoding the predicted KGF amino acid sequence. Nucleotides are numbered from the left; amino acids are numbered throughout. The NH_2-terminal peptide sequence derived from purified KGF is underlined. The hydrophobic NH_2-terminal domain is shown in italics. The potential asparagine-linked glycosylation site is overlined. **C:** Identification of KGF mRNAs by RNA blot analysis. An RNA blot of poly(A)$^+$-selected M426 RNA was hybridized with a ^{32}P-labeled 695-bp Bam HI-Bcl I fragment from clone 32 (probe A in A), lane a, or a 872-bp fragment from the 3′ untranslated region of clone 49 (probe B in A) generated by the polymerase chain reaction technique.

by RNA blot analysis of polyadenylated [poly(A)$^+$] M426 RNA (FIGURE 2C).[12] A probe derived from the 3′ untranslated region of clone 49, distal to the end of clone 32 (FIGURE 2A, probe B), only hybridized to the larger message (FIGURE 2C). Thus, it appears that the KGF gene is transcribed as two alternative mRNAs. Two other members of the FGF gene family, bFGF[32,33] and *int-2*,[36] also express multiple RNAs. The 3′ untranslated region of the 5-kb KGF cDNA contained many ATTTA sequences, which have been proposed to be markers for the selective degradation of transiently expressed, unstable RNAs[37] and might in part account for the low abundance of the larger KGF transcript.

To investigate the functional role of KGF, we examined the expression of its transcript in a variety of human cell lines and tissues. The predominant 2.4-kb KGF transcript was detected in each of several stromal fibroblast lines derived from epithelial tissues of embryonic, neonatal, and adult sources. In contrast, the transcript was not detected in normal glial cells, or in a variety of epithelial cell lines. The transcript was also evident in RNA extracted from normal adult kidney and organs of the gastrointestinal (GI) tract, but not from lung or brain.[12]

To further explore the stromal pattern of KGF expression, whole skin tissue was dissected from newborn mice and separated into dermal and epidermal layers by mild tryptic digestion.[6] Total cellular RNA was extracted from each layer, as well as from whole skin, and screened for KGF expression. The KGF transcript was observed in whole skin and was specifically detected in the dermis but not in the epidermal layer (FIGURE 3).[12] As controls for the enrichment for each tissue layer, we used DNA probes for vimentin[38,39] and keratin 1,[40,41] which are specific for mesenchymal and epithelial cells, respectively. The striking specificity of KGF RNA expression in stromal cells from epithelial tissues supports the concept that this factor is important in the normal mesenchymal stimulation of epithelial cell growth.

BIOCHEMICAL CHARACTERIZATION OF A HIGH-AFFINITY RECEPTOR FOR KGF: EVIDENCE FOR MULTIPLE FGF RECEPTORS

Consistent with its target cell specificity in mitogenesis bioassays, we detected saturable, specific high-affinity binding of ^{125}I-KGF to the surface of BALB/MK but not NIH/3T3 cells (FIGURE 4A and B). ^{125}I-KGF binding on BALB/MK was competed efficiently by aFGF but with 20-fold lower efficiency by bFGF (FIGURE 4A), in agreement with their relative potency in assays of DNA synthesis (FIGURE 5). The contrast in binding properties of BALB/MK and NIH/3T3 cells was reinforced by the pattern of ^{125}I-aFGF-receptor interactions on the two cell types. ^{125}I-aFGF exhibited specific high-affinity binding to both the keratinocytes and fibroblasts, but KGF competed only for the binding to BALB/MK. On the other hand, bFGF was a significantly better competitor of aFGF binding on NIH/3T3 compared to BALB/MK cells (FIGURE 4C and D).

Scatchard analysis of ^{125}I-KGF binding suggested major and minor high-affinity receptor components (dissociation constant = 400 and 25 pM, respectively) as well as a third high-capacity/low-affinity heparinlike component. The latter was also present on NIH/3T3 cells, and therefore must be insufficient for KGF-induced mitogenic signal transduction. Covalent affinity cross-linking of ^{125}I-KGF revealed two species of 115 and 140 kDa on BALB/MK cells which were absent from NIH/3T3 cells, and presumably corresponded to the high-affinity receptors required

poly(A)$^+$] M426 RNA (FIGURE 2C).[12] A
ion of clone 49, distal to the end of clone
o the larger message (FIGURE 2C). Thus,
d as two alternative mRNAs. Two other
and *int-2*,[36] also express multiple RNAs.
KGF cDNA contained many ATTTA
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nd might in part account for the low

GF, we examined the expression of its
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supports the concept that this factor is
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OF A HIGH-AFFINITY RECEPTOR ULTIPLE FGF RECEPTORS

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for mitogenic signaling. KGF also stimulated the rapid tyrosine phosphorylation of a 90-kDa protein in BALB/MK cells but not in NIH/3T3 fibroblasts. Tyrosine phosphorylation following addition of growth factor and, in particular, the prompt labeling of a 90-kDa substrate has also been observed in response to aFGF and

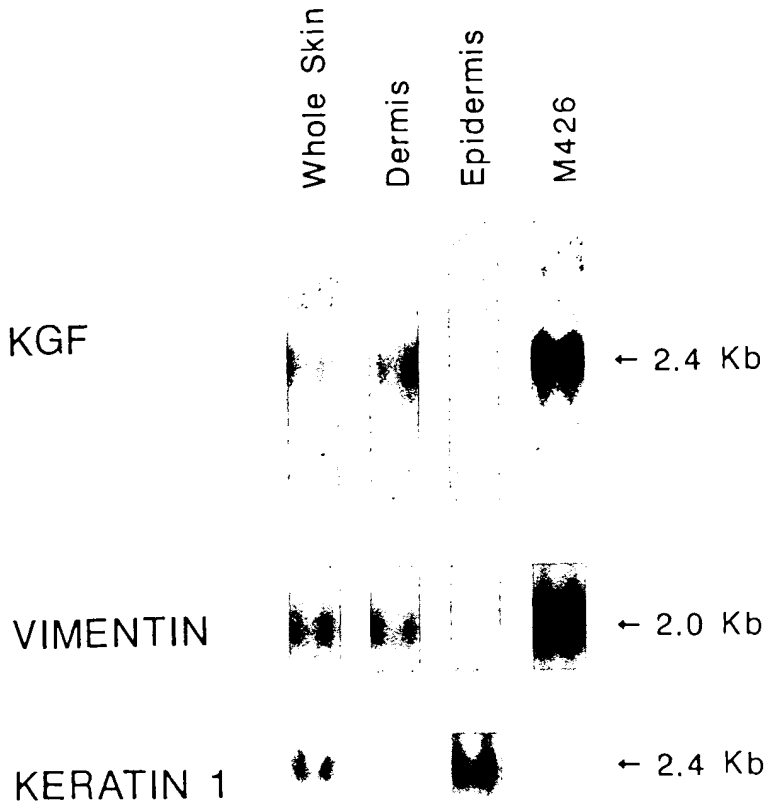


FIGURE 3. Expression of KGF in the skin of newborn mice. Whole skin was removed from 1-day-old mice and incubated overnight at 4°C in 0.25% trypsin solution. On the following day the dermal and epidermal layers were separated and RNA was extracted from these two layers as well as from intact mouse skin. RNA (20 μg) from each specimen, including the human fibroblast line M426, was screened for KGF transcript by RNA blot analysis with a ^{32}P-labeled Pvu II-Ssp I fragment of the human KGF cDNA (nucleotides 162 to 1380). Detection of vimentin and keratin I (K1) transcripts was done with human vimentin and mouse K1 cDNA derived probes. The arrows indicate the location of the transcript detected by each probe.

bFGF.[42–44] Together these results indicated that BALB/MK keratinocytes possess high-affinity KGF receptors to which the FGFs also bind, with aFGF having a greater affinity than bFGF. However, these receptors are distinct from the receptor(s) for aFGF and bFGF on NIH/3T3 fibroblasts, which fail to interact with KGF.

EXPRESSION cDNA CLONING OF THE KGF RECEPTOR BY CREATION OF A TRANSFORMING AUTOCRINE LOOP

Earlier studies, either with FGF family members such as *hst*/K-FGF[26,27] or FGF-5[25] that possess signal peptides, or with bFGF constructs to which a signal

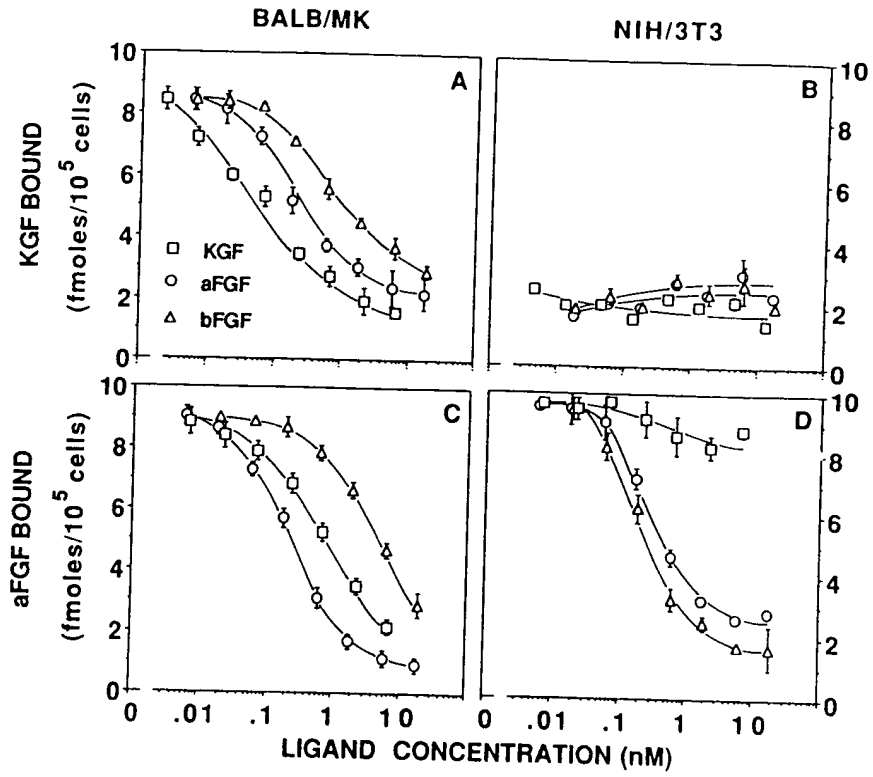
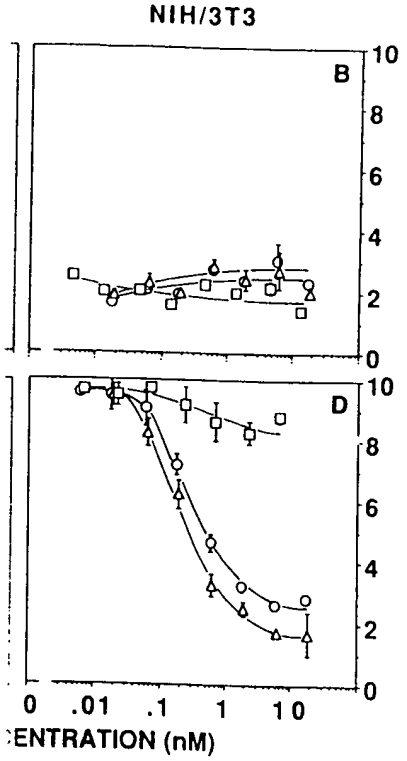


FIGURE 4. **A:** Specific binding of ^{125}I-KGF (1 ng/ml) to Balb/MK cells, expressed as femtomoles bound per 10^5 cells, competed by increasing concentrations (nM) of unlabeled KGF (squares), aFGF (circles), or bFGF (triangles). Values shown are the mean of triplicate samples ± standard deviation (SD). Where no error bars are shown, the error is less than the symbol size. Similar results were obtained using either low concentrations of heparin (1–3 μg/ml) or brief salt extraction to block low-affinity ligand binding in all competition studies shown. **B:** Specific ^{125}I-KGF binding on NIH/3T3 cells, competed by unlabeled KGF, aFGF, or bFGF. **C:** Specific binding of ^{125}I-aFGF (1 ng/ml) to Balb/MK cells, competed by unlabeled KGF, aFGF, or bFGF. **D:** Specific ^{125}I-aFGF binding on NIH/3T3 cells, competed by unlabeled KGF, aFGF, or bFGF.

peptide sequence had been added,[45,46] demonstrated that transfection of NIH/3T3 cells with vectors encoding these secreted factors would result in transformation. The presumed mechanism for this effect was the creation of a functional transforming autocrine loop. As indicated above, NIH/3T3 cells lack the high-affinity KGF receptors required to mediate mitogenic signal transduction. However, these cells,

E KGF RECEPTOR BY CREATION AUTOCRINE LOOP

y members such as *hst*/K-FGF[26,27] or th bFGF constructs to which a signal



nl) to Balb/MK cells, expressed as femto- g concentrations (nM) of unlabeled KGF Values shown are the mean of triplicate r bars are shown, the error is less than the either low concentrations of heparin (1–3 ligand binding in all competition studies lls, competed by unlabeled KGF, aFGF, or to Balb/MK cells, competed by unlabeled on NIH/3T3 cells, competed by unlabeled

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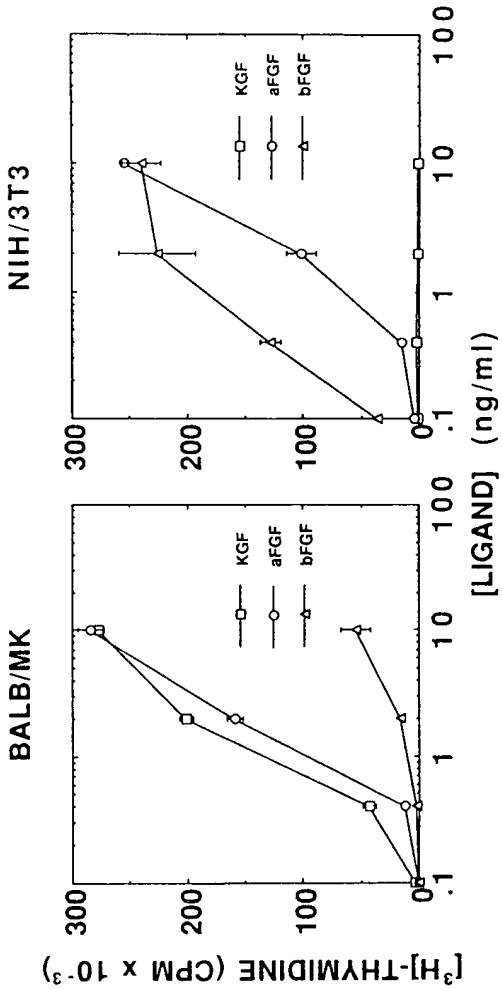

FIGURE 5. Comparison of BALB/MK and NIH/3T3 DNA synthesis in response to KGF, aFGF, and bFGF. Incorporation of [^{3}H]thymidine into trichloroacetic acid–insoluble DNA was measured as a function of the concentration of the indicated growth factors. The assays were performed in the absence of heparin, using bacterially expressed human KGF and bovine-brain-derived aFGF and bFGF. The results are mean values ± standard deviation of triplicate measurements.

like other fibroblast lines, synthesize and secrete KGF.[14] Thus, we reasoned that the introduction of KGF receptors via transfection might cause transformation by creation of a similar, KGF-dependent autocrine mechanism. Screening foci of transformed cells for evidence of high-affinity KGF binding sites would enable us to identify those cells that had become transformed as a consequence of acquiring such an autocrine loop.

This approach was successfully carried out with a newly designed expression cDNA cloning vector that incorporated the attributes of stable transfection of long cDNA inserts with a high cloning efficiency and the capacity for plasmid rescue.[47] When a cDNA library prepared with mRNA from BALB/MK cells was introduced into NIH/3T3 using this vector, 15 transformed foci were detected which were associated with three distinct morphological phenotypes. A single cDNA clone rescued from each transformant was found to possess high-titered transforming activity ranging from 10^3 to 10^4 focus-forming units per nanomole of DNA. Transfectants induced by the individual plasmids containing these epithelial-cell-transforming cDNAs (designated *ect1, ect2,* and *ect3*) were used in subsequent analyses.

To investigate the possibility that any of the three genes might encode the KGF receptor, we performed binding studies with recombinant ^{125}I-labeled KGF. BALB/MK cells showed specific high-affinity binding of ^{125}I-labeled KGF, which was not observed when NIH/3T3 cells were used. Expression of the *ect1* gene by NIH/3T3 cells resulted in the acquisition of 3.5-fold more ^{125}I-labeled KGF binding sites than BALB/MK cells (FIGURE 6). Under the same conditions, control NIH/3T3 as well as transfectants containing either *ect2* or *ect3* did not bind the labeled growth factor. These results suggested that *ect1* encoded the KGF receptor (KGFR), whose introduction into NIH/3T3 cells had completed a transforming autocrine loop.

A single *ect1* transcript of around 4.2 kb was observed in BALB/MK cells. Thus, our cDNA clone of 4.2 kb represented essentially the complete *ect1* transcript.[14] In NIH/3T3 cells, a transcript of comparable size was only faintly detectable under stringent hybridization conditions. Therefore, if this transcript were to represent *ect1* rather than a related gene, its expression was markedly lower in fibroblasts as compared to epithelial cells.

Nucleotide sequence analysis of the 4.2-kb *ect1* cDNA revealed a long open reading frame of 2235 nucleotides (nucleotide position 562 to 2796). Two methionine codons were found at nucleotide positions 619 and 676, respectively. The second methionine codon matched the Kozak's consensus for a translational initiator sequence (A/GC-CATGG).[48] Moreover, it was followed by a characteristic signal sequence of 21 residues, 10 of which were identical to those of the putative signal peptide of the mouse basic FGF receptor.[49–51] Thus, it seems likely that the second ATG is the authentic initiation codon. If so, the receptor polypeptide would comprise 707 amino acids with a predicted size of 82.5 kD (FIGURE 7A).

The amino acid sequence predicted a transmembrane tyrosine kinase structurally related to but distinct from the mouse bFGF receptor (bFGFR) encoded by the *flg* gene. The percent similarity between both proteins is shown in FIGURE 7B. The putative KGFR extracellular portion contained two immunoglobulin (Ig) like domains, exhibiting 77% and 60% similarity with the Ig-like domains 2 and 3, respectively, of the mouse bFGFR. Studies have revealed a variant form of the bFGFR, in which the extracellular domain also contains only these two corresponding Ig-like domains.[50,51] The sequence NH_2 terminal to the first Ig-like domain of the KGFR was 63 residues long, compared to 88 residues found in the shorter form of the mouse bFGFR. Both chicken and mouse bFGFRs contain a stretch of eight

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rminal to the first Ig-like domain of the
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consecutive acidic residues between the first and second Ig-like domains[16-18] not found in the KGFR (FIGURE 7B).

The kinase domain of the KGFR was 90% related to the bFGFR tyrosine kinase (FIGURE 7B). The central core of the catalytic domain was flanked by a relatively long juxtamembrane sequence, and the tyrosine kinase domain was split by a short insert of 14 residues, similar to that observed in mouse, chicken, and human bFGF receptors.[49-55] Hanafusa and coworkers isolated a partial cDNA for a tyrosine kinase gene, designated *bek*, by bacterial expression cloning with phosphotyrosine antibodies.[56] The reported sequence of *bek* was identical to the KGFR in the tyrosine kinase domain (FIGURE 7B).

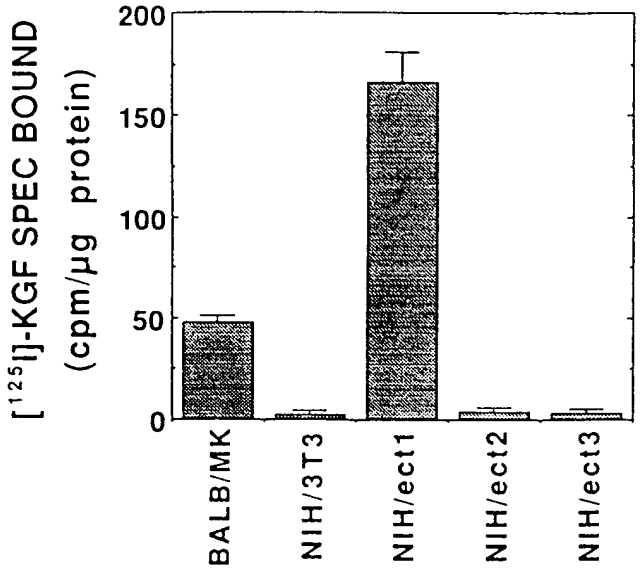

FIGURE 6. Comparison of [^{125}I]KGF specific binding to BALB/MK, NIH/3T3, and NIH/3T3-transfected cells expressing either of the epithelial-cell-transforming cDNAs designated *ect1*, *ect2*, and *ect3*. Bound cpm were normalized according to protein content of SDS extracts. Specific binding was determined by subtracting normalized cpm of samples incubated with a 100-fold excess of unlabeled KGF from the normalized cpm bound in the presence of [^{125}I]KGF alone. The results are mean values ± SD of triplicate measurements.

Scatchard analysis of ^{125}I-labeled KGF binding to the NIH/*ect1* transfectant revealed expression of high-affinity receptors comparable to the high-affinity KGF binding sites displayed by BALB/MK cells.[13,14] The pattern of KGF and FGF competition for ^{125}I-labeled KGF binding to NIH/*ect1* cells was also very similar to that observed with BALB/MK cells. When ^{125}I-labeled KGF cross-linking was performed with NIH/*ect1* cells, we observed a single species corresponding in size to the smaller 137-kDa complex in BALB/MK cells. Moreover, detection of this band was specifically and efficiently blocked by unlabeled KGF. When glycosylation is considered, the size of the KGFR predicted by sequence analysis corresponds reasonably well with the corrected size (115 kDa, taking into account the size of KGF) of the cross-linked KGFR in the *ect1* transfectant.[14]

A

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  1 MVSWGRFICLVLVTMATLSLARPSFSLVEDTTLEPEGAPYWTNTEKMEKRLHAVPAANTVKFRCPAGGNPTPTMRWL
 81 KEFKQEHRIGGYKVRNQHWSLIMESVVPSDKGNYTCLVENEYGSINHTYHLDVVERSPHRPILQAGLPANASTVVGG
161 FVCKVYSDAQPHIQWIKHVEKNGSKYGPDGLPYLKVLKHSGINSSNAEVLALFNVTEMDAGEYICKVSNYIGQANQS
241 TVLPKQQAPVREKEITASPDYLEIAIYCIGVFLIACMVVTVIFCRMKTTTKKPDFSSQPAVHKLTKRIPLRRQVTVS
321 SSSMNSNTPLVRITTRLSSTADTPMLAGVSEYELPEDPKWEFPRDKLTLGKPLGEGCFGQVVMAEAVGIDKDKPKEA
401 AVKMLKDDATEKDLSDLVSEMEMMKMIGKHKNIINLLGACTQDGPLYVIVEYASKGNLREYLRARRPPGMEYSYDIN
481 EEQMTFKDLVSCTYQLARGMEYLASQKCIHRDLAARNVLVTENNVMKIADFGLARDINNIDYYKKTTNGRLPVKWMA
561 LFDRVYTHQSDVWSFGVLMWEIFTLGGSPYPGIPVEELFKLLKEGHRMDKPTNCTNELYMMMRDCWHAVPSQRPTFK
641 EDLDRILTLTTNEEYLDLTQPLEQYSPSYPDTRSSCSSGDDSVFSPDPMPYEPCLPQYPHINGSVKT
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B

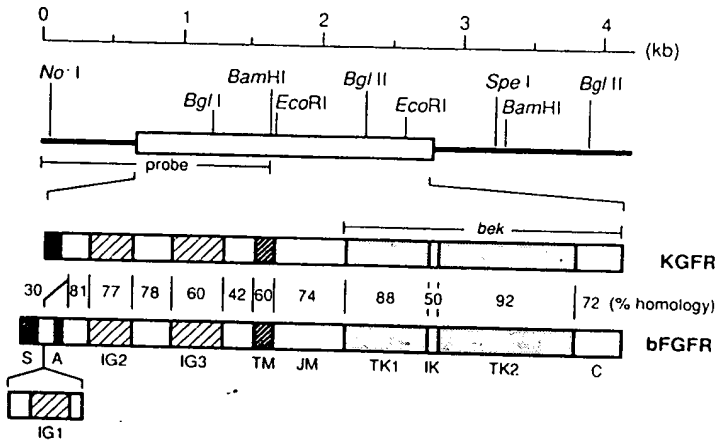
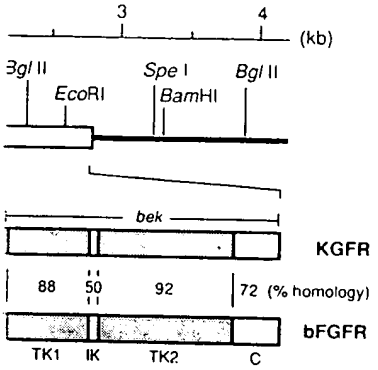


FIGURE 7. Primary structure of the KGF receptor. **A:** Amino acid sequence deduced from the coding region of the KGF receptor cDNA. Amino acids are numbered from the putative initiation site of translation. Potential sites of N-linked glycosylation are underlined. The potential signal peptide and transmembrane domains are boxed. The interkinase domain is shown by underlined italic letters. Glycine residues considered to be involved in ATP (adenosine triphosphate) binding are indicated by asterisks. Cysteine residues delimit two Ig-like domains in the extracellular portion of the molecule shown by boldface. Nucleotide sequence was determined by the chain termination method. **B:** Structural comparison of the predicted KGF and bFGF receptors. The region used as a probe for Southern and Northern analysis (Figure 1B and C) is indicated. The region homologous to the published *bek* sequence[56] is also shown. The schematic structure of KGF receptor is shown below the restriction map of the cDNA clone. Amino acid sequence similarities with the smaller and larger bFGF receptor variants are indicated. S, signal peptide; A, acidic region; IGI, IG2, and IG3, Ig-like domains; TM, transmembrane domain; JM, juxtamembrane domain; TK1 and TK2, tyrosine kinase domains; IK, interkinase domain; C, COOH-terminus domain.

Our expression cloning of the KGFR was based on its transforming activity for NIH/3T3 cells that synthesize KGF. Thus, its detection could reflect activation of an autocrine loop involving KGF and the normal receptor. Alternatively, the cDNA might have been detected fortuitously as a constitutively activated KGFR mutant. Suramin, which interferes with ligand-receptor interactions,[57,58] inhibited DNA synthesis of KGFR transfectants. We also observed specific inhibition of proliferation of such cells in response to a KGF monoclonal antibody, which neutralizes KGF mitogenic activity.[14] Together these findings argue that induction of the transformed

DAPYWTNTEKMEKRLHAVPAANTVKFRCPAGGNPTPTMRWLKNG
LVENEYGSINHTYHLDVVERSPHRPILQAGLPANASTVVGGDVE
KHSGINSSNAEVLALFNVTEMDAGEYICKVSNYIGQANQSAWL
TVVTVIFCRMKTTTKKPDFSSQPAVHKLTKRIPLRRQVTVSAES
PKWEFPRDKLTLGKPLGEGCFGQVVMAEAVGIDKDKPKEAVTV
GACTQDGPLYVIVEYASKGNLREYLRARRPPGMEYSYDINRVP
EVLVTENNVMKIADFGLARDINNIDYYKKTTNGRLPVKWMAPEA
LFKLLKEGHRMDKPTNCTNELYMMMRDCWHAVPSQRPTFKQLV
EDDSVFSPDPMPYEPCLPQYPHINGSVKT



ır. **A:** Amino acid sequence deduced from the ino acids are numbered from the putative N-linked glycosylation are underlined. The ains are boxed. The interkinase domain is s considered to be involved in ATP (adeno- risks. Cysteine residues delimit two Ig-like ule shown by boldface. Nucleotide sequence l. **B:** Structural comparison of the predicted probe for Southern and Northern analysis ogous to the published *bek* sequence[56] is also r is shown below the restriction map of the with the smaller and larger bFGF receptor region; IGI, IG2, and IG3, Ig-like domains; ne domain; TK1 and TK2, tyrosine kinase inus domain.

as based on its transforming activity for s detection could reflect activation of an nal receptor. Alternatively, the cDNA constitutively activated KGFR mutant. tor interactions,[57,58] inhibited DNA syn- ed specific inhibition of proliferation of nal antibody, which neutralizes KGF argue that induction of the transformed phenotype resulted from autocrine KGF stimulation of an ectopically expressed normal KGFR cDNA.

There have been reports concerning human or avian cDNAs closely related to the KGFR.[59–62] The external portions of *bek* (human) and *cek3* (chicken) proteins contain three Ig-like domains.[59–61] These molecules also differ from the KGFR in that each contains an acidic region and is completely divergent in the COOH-terminal half of its third Ig-like domain from the KGFR. Binding studies with the three Ig-like domain human *bek* variant have indicated similar high affinities for aFGF and bFGF.[59] Since the affinity of the KGFR for aFGF was substantially higher than for bFGF, differences in FGF binding by these receptor molecules must relate to these regions of divergence. In BALB/MK cells, we detected a higher molecular weight KGF-cross-linked species, corresponding in size to the three Ig-like domain *bek* variant.[13] Whether it represents this variant or the product of a distinct gene remains to be determined.

A gene, designated K-*sam,* was recently identified as an amplified sequence in a human stomach carcinoma.[62] A cDNA clone corresponding to one of the overexpressed K-*sam* transcripts predicts a two Ig-like domain *bek* variant, whose Ig-like domains correspond to those of the KGFR. However, it differs in that it contains an acidic region and may be truncated at its COOH terminus as well.[62] These molecules likely reflect alternative transcripts of the same gene, as has also been suggested for two and three Ig-like domain forms of the bFGFR.[63]

SUMMARY AND FUTURE DIRECTIONS

KGF is a fibroblast-derived member of the FGF family, with potent mitogenic activity on epithelial cells but no corresponding activity on fibroblasts, endothelial cells, melanocytes, or other nonepithelial targets of FGF action. Biochemical analysis established that KGF receptors bound aFGF with a high affinity but bFGF with at least an order of magnitude lower affinity. Expression cDNA cloning of a KGF receptor was accomplished by creation of a transforming autocrine loop. The full-length cDNA encoded a transmembrane, tyrosine kinase molecule which resembled the bFGF receptor encoded by *flg,* and was even more similar to the *bek* gene product. Future study will be aimed at determining differences responsible for the binding specificities that distinguish the KGF receptor from the *bek* and *flg* gene products. Using molecular probes to both KGF and its receptor to study their expression during development and in the adult should help define their role in normal growth and repair processes as well as possible pathologic roles in disease. This information, along with experiments testing the effects of KGF *in vivo,* could serve to identify situations in which KGF or antagonists to its actions would be of therapeutic benefit.

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Biochemistry

EXHIBIT 8

Purification and characterization of a newly identified growth factor specific for epithelial cells

(mitogen/keratinocyte/protein purification/heparin binding/N-terminal sequence)

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ABSTRACT **A growth factor specific for epithelial cells was identified in conditioned medium of a human embryonic lung fibroblast cell line. The factor, provisionally termed keratinocyte growth factor (KGF) because of its predominant activity on this cell type, was purified to homogeneity by a combination of ultrafiltration, heparin-Sepharose affinity chromatography, and hydrophobic chromatography on a C_4 reversed-phase HPLC column. KGF was both acid and heat labile and consisted of a single polypeptide chain of ≈28 kDa. Purified KGF was a potent mitogen for epithelial cells, capable of stimulating DNA synthesis in quiescent BALB/MK epidermal keratinocytes by >500-fold with activity detectable at 0.1 nM and maximal at 1.0 nM. Lack of mitogenic activity on either fibroblasts or endothelial cells indicated that KGF possessed a target cell specificity distinct from any previously characterized growth factor. Microsequencing revealed an amino-terminal sequence containing no significant homology to any known protein. The release of this growth factor by human embryonic fibroblasts raises the possibility that KGF may play a role in mesenchymal stimulation of normal epithelial cell proliferation.**

Growth factors are important mediators of intercellular communication. These potent molecules are generally released by one cell type and act to influence proliferation of other cell types (1). Interest in growth factors has been heightened by evidence of their potential involvement in neoplasia. The v-*sis* transforming gene of simian sarcoma virus encodes a protein that is homologous to the B chain of platelet-derived growth factor (2, 3). Moreover, a number of oncogenes are homologues of genes encoding growth factor receptors (4). Thus, increased understanding of growth factors and their receptor-mediated signal-transduction pathways is likely to provide insights into mechanisms of both normal and malignant cell growth.

Recognizing that the vast majority of human malignancies are derived from epithelial tissues (5), we sought to identify growth factors specific for these cell types. In this communication, we report the purification to homogeneity of such a growth factor released by a human embryonic lung fibroblast line. Our demonstration of its unique N-terminal amino acid sequence and epithelial cell specificity distinguishes this mitogen from any previously described growth factor.

METHODS AND MATERIALS

Cell Culture. M426 human embryonic fibroblasts (6), BALB/MK mouse epidermal keratinocytes (7), and NIH 3T3 mouse embryonic fibroblasts (8) were established in this laboratory. CCL208 rhesus monkey bronchial epithelial cells (9) were obtained from the American Type Culture Collection, and the B5/589 human mammary epithelial cell line, prepared as described (10), was a gift from M. Stampfer (Lawrence Berkeley Laboratory). Primary cultures of human saphenous vein endothelial cells were prepared and maintained as described elsewhere (11). Epidermal growth factor (EGF) and insulin were from Collaborative Research, and transforming growth factor type α (TGF-α) was from Genentech. Acidic fibroblast growth factor (aFGF) and basic FGF (bFGF) were gifts from J. Abraham (California Biotechnology, Inc.). Media and serum were from GIBCO, Biofluids (Rockville, MD), or the National Institutes of Health media kitchen.

Preparation of Conditioned Medium. An early passage of M426 fibroblasts was plated onto 175-cm^2 T flasks and grown to confluence over 10–14 days in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% (vol/vol) calf serum (GIBCO). Once confluent, the monolayers were cycled weekly from serum-containing to serum-free medium, the latter consisting of DMEM alone. The cells were washed twice with 5 ml of phosphate-buffered saline prior to addition of 20 ml of DMEM. After 72 hr, culture fluids were collected and replaced with 35 ml of serum-containing medium. The conditioned medium was stored at −70°C until further use.

Ultrafiltration. Approximately 10 liters of conditioned medium was thawed, prefiltered through a 0.50-μm filter (Millipore HAWP 142 50), and concentrated to 200 ml by using the Pellicon cassette system (Millipore XX42 00K 60) and a cassette having a 10-kDa molecular mass cutoff (Millipore PTGC 000 05). After concentration, the sample was subjected to two successive rounds of dilution with 1 liter of 20 mM Tris·HCl, pH 7.5/0.3 M NaCl, each followed by ultrafiltration with the Pellicon system. Activity recovered in the retentate was either immediately applied to the heparin-Sepharose resin or stored at −70°C.

Heparin-Sepharose Affinity Chromatography (HSAC). The retentate from ultrafiltration was loaded onto heparin-Sepharose resin (Pharmacia) that had been equilibrated in 20 mM Tris·HCl, pH 7.5/0.3 M NaCl. The resin was washed extensively until the absorbance had returned to baseline and then was subjected to a linear-step gradient of increasing NaCl concentration. After aliquots were removed from the fractions for the thymidine incorporation bioassay, selected fractions were concentrated 10- to 20-fold with a Centricon-10 microconcentrator (Amicon) and stored at −70°C.

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Abbreviations: KGF, keratinocyte growth factor; EGF, epidermal growth factor; TGF-α, transforming growth factor α; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HSAC, heparin-Sepharose affinity chromatography; RP-HPLC, reversed-phase HPLC.

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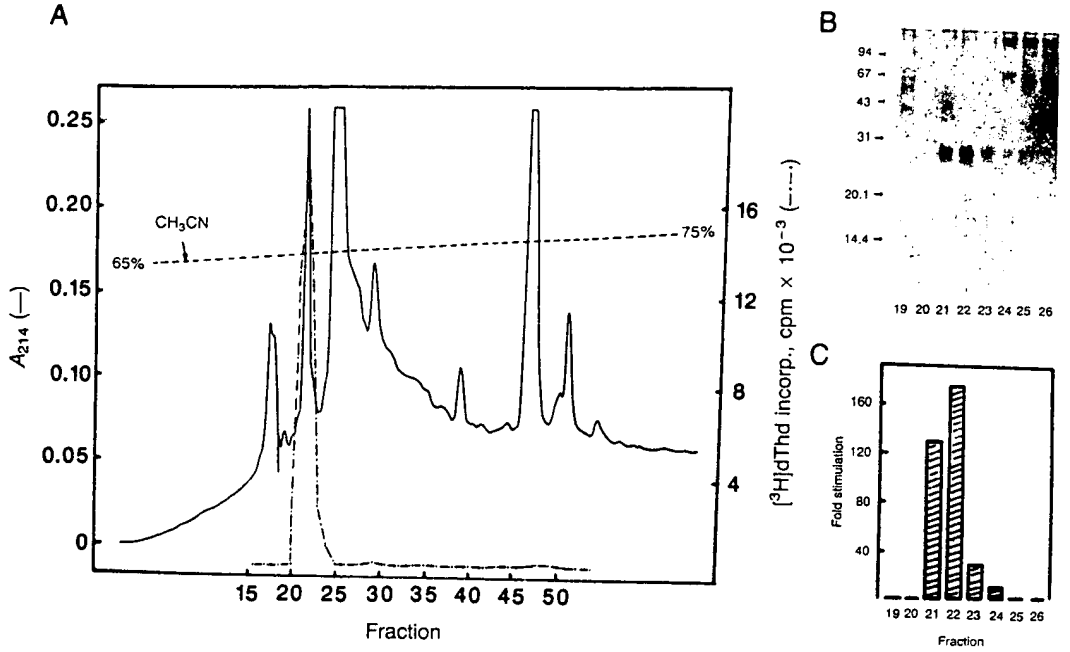

FIG. 2. (*A*) C_4 RP-HPLC of BALB/MK mitogenic activity. Active fractions eluted from the heparin-Sepharose column with 0.6 M NaCl were processed with the Centricon-10 microconcentrator and loaded directly onto a C_4 Vydac column (4.6 × 250 mm) that had been equilibrated in 0.1% trifluoroacetic acid/20% acetonitrile. After the column was washed with 4 ml of equilibration buffer, the sample was eluted with a modified linear gradient of increasing percentage of acetonitrile. Fraction size was 0.2 ml, and flow rate was 0.5 ml/min. Aliquots for the assay of [3H]thymidine incorporation (incorp.) in BALB/MK cells were promptly diluted 1:10 with 50 μg of bovine serum albumin per ml/20 mM Tris·HCl, pH 7.5, and tested at a final dilution of 1:200. (*B*) $NaDodSO_4$/PAGE analysis of selected fractions from the C_4 chromatography shown in *A*. Half of each fraction was dried, redissolved in $NaDodSO_4$/2-mercaptoethanol, heat-denatured, and electrophoresed in a 14% polyacrylamide gel which was subsequently silver-stained. The position of each molecular mass marker (in kDa) is indicated by an arrow. (*C*) DNA synthesis in BALB/MK cells triggered by the fractions analyzed in *B*. Activity is expressed as the fold stimulation over background, which was 100 cpm.

judged by silver-stained $NaDodSO_4$/PAGE (data not shown) but provided a far better recovery of activity (Table 1). The TSK-purified material was used routinely for biological studies as it had a higher specific activity. In both instances, the profile of mitogenic activity was associated with a distinct band on $NaDodSO_4$/PAGE that appeared to be indistinguishable in the two preparations.

Physical and Biological Characterization of the Growth Factor. The purified factor had an estimated molecular mass of 28 kDa based on $NaDodSO_4$/PAGE under reducing (Fig. 2) and nonreducing conditions (data not shown). This value was in good agreement with its elution position on two different sizing columns run in solvents expected to maintain native conformation [TSK G3000SW (Fig. 3) and Superose-12 (data not shown)]. From these data, the mitogen appears to consist of a single polypeptide chain with a molecular mass of 25–30 kDa.

Its heat and acid lability were demonstrated by using the BALB/MK mitogenesis bioassay. While activity was unaffected by a 10-min incubation at 50°C, it was reduced by 68% after 10 min at 60°C and was undetectable after 3 min at 100°C. Exposure to 0.5 M acetic acid for 60 min at room temperature resulted in a decline in activity to 14% of the control. In comparison, the mitogenic activity of EGF was not diminished by any of these treatments.

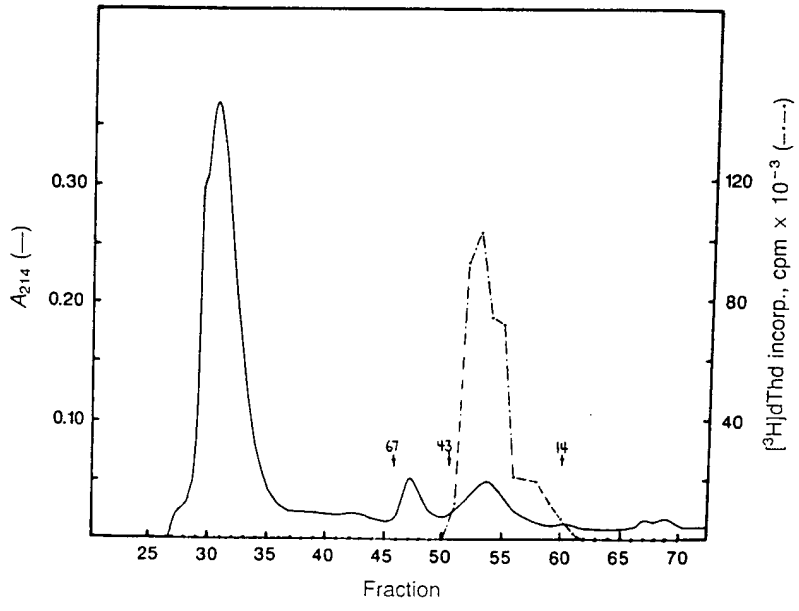

FIG. 3. TSK G3000SW chromatography of the BALB/MK mitogenic activity. Approximately 50 μl of a Centricon microconcentrator-processed 0.6 M NaCl pool from HSAC was loaded onto a GlasPac TSK G3000SW column (8 × 300 mm), previously equilibrated in 20 mM Tris·HCl, pH 6.8/0.5 M NaCl, and was eluted as 0.2-ml fractions at a flow rate of 0.4 ml/min. Aliquots of 2 μl were transferred to microtiter wells and diluted to a final volume of 0.2 ml for assay of [^{3}H]thymidine incorporation (incorp.) in BALB/MK cells. The elution positions of molecular mass markers (in kDa) were as indicated by the arrows.

Table 1. Growth-factor purification

Purification step	Protein, mg	Total activity,* units	Specific activity,* units/mg
Conditioned medium (10 liters)	1.4×10^3†	2.5×10^4	1.8×10^1
Ultrafiltration retentate	1.3×10^3†	3.2×10^4	2.5×10^1
HSAC 0.6 M NaCl pool	0.73‡	1.6×10^4	2.2×10^4
TSK G3000SW	8.4×10^{-3}‡	2.7×10^3	3.2×10^5
C_4 HPLC	6.1×10^{-3}‡	2.1×10^2	3.4×10^4

Recoveries were calculated by assuming that all of the mitogenic activity in the starting material was due to the isolated factor.
*One unit of activity is defined as half of the maximal stimulation of thymidine incorporation induced by TSK-purified growth factor in the BALB/MK bioassay. Approximately 3 ng of the TSK-purified factor stimulated 1 unit of activity in this bioassay.
†Protein was estimated by using the Bradford reagent from Bio-Rad (23).
‡Protein was estimated by using $A_{214}^{1\%} = 140$.

The dose–response curve for the purified growth factor depicted in Fig. 4 illustrates that as little as 0.1 nM led to a detectable stimulation of DNA synthesis. Thus, the activity range was comparable to that of the other growth factors analyzed to date. A linear relationship was observed in the concentration range 0.1–1.0 nM, with maximal stimulation of 600-fold observed at 1.0 nM. The novel factor consistently induced a higher level of maximal thymidine incorporation than did EGF, aFGF, or bFGF (Fig. 4).

Its distinctive target-cell specificity was demonstrated by comparing it on a variety of cell types with other growth factors known to possess epithelial cell mitogenic activity. The newly isolated factor exhibited a strong mitogenic effect on BALB/MK cells and induced demonstrable thymidine incorporation in the other epithelial cells tested (Table 2). In striking contrast, the factor had no detectable effects on mouse (or human, data not shown) fibroblasts or human

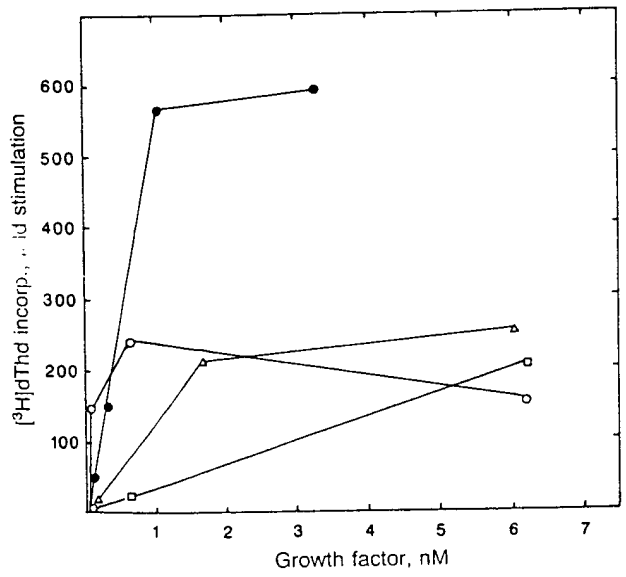

FIG. 4. Comparison of BALB/MK DNA synthesis in response to TSK-purified mitogen and other growth factors. Incorporation (incorp.) of [^{3}H]thymidine into trichloroacetic acid-insoluble DNA, expressed as fold stimulation over background, was measured as a function of the concentration of the indicated growth factors. The background value with no sample added was 150 cpm. The results represent mean values of two independent experiments. Replicates in each experiment were within 10% of mean values. ●, TSK-purified mitogen; △, EGF; □, aFGF; ○, bFGF.

Table 2. Target-cell specificity of growth factors

Growth factor	Fold stimulation of thymidine incorporation				
	Epithelial cell line			Fibroblast	Endothelial cell line†
	BALB/MK	B5/589*	CCL208	NIH 3T3S	
KGF	500–1000	2–3	5–10	<1	<1
EGF	100–200	20–40	10–30	10–20	ND
TGF-α	150–300	ND	ND	10–20	ND
aFGF‡	300–500	2–3	5–10	50–70	5
bFGF	100–200	2–3	2–5	50–70	5

Comparison of maximal thymidine incorporation stimulated by KGF and other growth factors in a variety of cell lines, expressed as fold stimulation over background. These data represent a summary of four different experiments. ND, not determined.
*The mammary cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 4 ng of EGF per ml. When maintained in serum-free conditions, the basal medium was DMEM.
†Human saphenous vein cells.
‡Maximal stimulation by aFGF required the presence of heparin (Sigma) at 20 μg/ml.

saphenous vein endothelial cells. By comparison, TGF-α and EGF showed good activity on fibroblasts, while aFGF and bFGF were mitogenic for endothelial cells as well (Table 2). Because of its specificity for epithelial cells and the sensitivity of keratinocytes in particular, the mitogen was provisionally designated "keratinocyte growth factor" (KGF).

To establish that KGF not only would stimulate DNA synthesis but also would support sustained cell growth, we attempted to grow BALB/MK cells in a fully defined, serum-free medium supplemented with this growth factor. KGF served as an excellent substitute for EGF but not for insulin (or insulin-like growth factor I) in this chemically defined medium (Fig. 5). Thus, KGF acts through the major signaling pathway shared by EGF, aFGF, and bFGF for proliferation of BALB/MK cells (14).

Microsequencing Reveals a Unique N-terminal Amino Acid Sequence of KGF. To further characterize the growth factor, ≈150 pmol of C_4-purified material was subjected to sequence analysis. A single sequence was detected with unambiguous assignments made for cycles 2–13 as follows: Xaa-

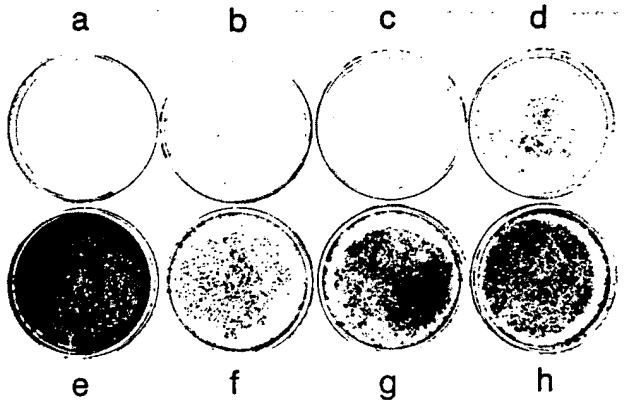

FIG. 5. Comparative growth of BALB/MK cells in a chemically defined medium in response to different combinations of growth factors. Cultures were plated at a density of 2.5×10^4 cells per dish on poly(D-lysine)/fibronectin-precoated 35-mm Petri dishes in 1:1 (vol/vol) Eagle's minimal essential medium/Ham's F-12 medium supplemented with transferrin, Na_2SeO_3, ethanolamine, and the growth factors indicated below. After 10 days, the plates were fixed and stained with Giemsa. (*a*) No growth factor. (*b*) EGF alone. (*c*) Insulin alone. (*d*) KGF alone. (*e*) EGF and dialyzed fetal calf serum (final concentration, 10%). (*f*) KGF and EGF. (*g*) KGF and insulin. (*h*) EGF and insulin. Final concentrations of the growth factors were as follows: EGF, 20 ng/ml; insulin, 10 μg/ml; and KGF, 40 ng/ml.

Reversed-Phase HPLC (RP-HPLC). Active fractions (0.6 M NaCl pool) from the HSAC were thawed, pooled, and further concentrated with the Centricon-10 microconcentrator to a final volume of ≤200 μl. The sample was loaded onto a Vydac C_4 HPLC column (The Separations Group) that had been equilibrated in 0.1% trifluoroacetic acid (Fluka)/20% acetonitrile (Baker, HPLC grade) and eluted with a linear gradient of increasing acetonitrile. Aliquots for the bioassay were immediately diluted in a 10-fold excess of 20 mM Tris·HCl (pH 7.5) containing bovine serum albumin (fraction V, Sigma) at 50 μg/ml. The remainder of the sample was dried in a Speed-Vac (Savant) in preparation for structural analysis.

Molecular-Sieve HPLC. Approximately 50 microliters of the twice concentrated heparin-Sepharose fractions were loaded onto a TSK G3000SW Glas-Pac column (LKB) that had been equilibrated in 20 mM Tris·HCl, pH 6.8/0.5 M NaCl. The sample was eluted in this buffer at a flow rate of 0.4 ml/min. After removing aliquots for the bioassay, the fractions were stored at −70°C.

$NaDodSO_4$/PAGE. Polyacrylamide gels were prepared with $NaDodSO_4$ by the procedure of Laemmli (12). Samples were boiled for 3 min in the presence of 2.5% (vol/vol) 2-mercaptoethanol. The gels were fixed and silver-stained (13) by using the reagents and protocol from Bio-Rad. Molecular weight markers were from Pharmacia.

Mitogenic Assay. DNA synthesis was measured as described elsewhere (14) with a few modifications. Ninety-six-well microtiter plates (Falcon no. 3596) were precoated with human fibronectin (Collaborative Research) at 1 μg/cm² prior to seeding with indicator cells. Incorporation of [³H]-thymidine was monitored during a 6-hr period beginning 16 hr after addition of samples.

Proliferation Assay. Culture dishes (35 mm) were precoated sequentially with poly(D-lysine) (20 μg/cm²; Sigma) and human fibronectin and then were seeded with ≈2.5 × 10⁴ BALB/MK cells. The basic medium was 1:1 (vol/vol) Eagle's low-Ca^{2+} minimal essential medium/Ham's F-12 medium supplemented with 5 μg of transferrin per ml, 30 nM Na_2SeO_3, and 0.2 mM ethanolamine (Sigma). Medium was changed every 2 or 3 days. After 10 days, the cells were fixed in formalin (Fisher) and stained with Giemsa (Fisher).

Protein Microsequencing. Approximately 4 μg (≈150 pmol) of protein from the active fractions of the C_4 column were redissolved in 50% trifluoroacetic acid and loaded onto an Applied Biosystems gas-phase protein sequenator. Twenty rounds of Edman degradation were carried out, and identifications of amino acid derivatives were made with an automated on-line HPLC column (model 120A, Applied Biosystems).

RESULTS

Growth Factor Detection and Isolation. Preliminary screening of conditioned media from various cell lines indicated that media from some fibroblast lines contained mitogenic activities detectable on both BALB/MK epidermal keratinocytes and NIH 3T3 embryonic fibroblasts. Whereas boiling destroyed the activity on BALB/MK cells, mitogenic activity on NIH 3T3 cells remained intact. Based on the known heat stability of EGF (15) and TGF-α (16), we reasoned that BALB/MK mitogenic activity might be due to a different agent. M426, a human embryonic lung fibroblast line, was selected as the best source of this activity for purification of the putative growth factor(s).

Ultrafiltration with the Pellicon system provided a convenient way of reducing the sample volume to a suitable level for subsequent chromatography. HSAC, which has been used in the purification of other growth factors (17–22), provided the most efficient purification step. While estimates of specific recovered activity were uncertain at this stage because of the likely presence of other factors, the apparent yield of activity was 50–70% with a corresponding enrichment of ≈1000-fold. More than 90% of the BALB/MK mitogenic activity was eluted with 0.6 M NaCl (Fig. 1) and was not associated with any activity on NIH 3T3 cells (data not shown). Because of the reproducibility of the HSAC pattern, active fractions could be identified presumptively on the basis of the gradient and absorption profile. Prompt concentration of 10- to 20-fold with the Centricon-10 microconcentrator was found to be essential for stability, which could be maintained subsequently at −70°C for several months.

Final purification was achieved by RP-HPLC with a C_4 Vydac column, a preparative method suitable for amino acid sequence analysis. While the yield of activity from the C_4 step was usually only a few percent, this loss could be attributed to the solvents used. In other experiments, exposure to 0.1% trifluoroacetic acid/50% (vol/vol) acetonitrile for 1 hr at room temperature reduced the mitogenic activity of the preparation by 98%. Nonetheless, a single peak of BALB/MK stimulatory activity was obtained (Fig. 2), coinciding with a distinct peak in the absorption profile. The peak fractions contained a single band on the silver-stained gel (Fig. 2*B*), and the relative mitogenic activity (Fig. 2*C*) correlated well with the intensity of the band across the activity profile.

An alternative step, using molecular-sieve chromatography with a TSK G3000SW GlasPac column run in aqueous solution near physiologic pH, resulted in a major peak of activity in the BALB/MK bioassay (Fig. 3). This preparation was almost as pure as the one obtained from RP-HPLC as

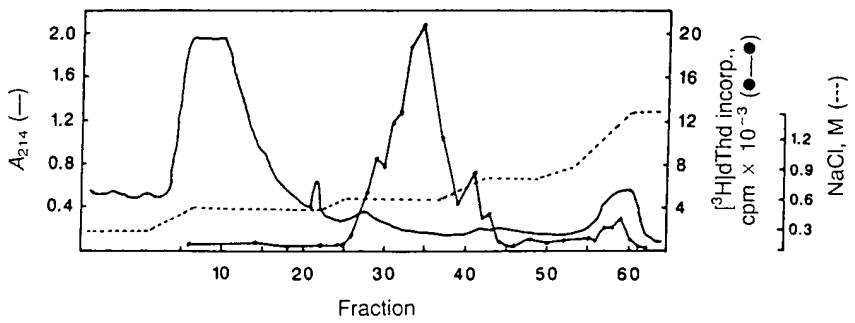

FIG. 1. HSAC of conditioned medium from M426 human embryonic fibroblasts. Approximately 150 ml of ultrafiltration retentate derived from 5 liters of M426-conditioned medium was loaded onto a heparin-Sepharose column (6-ml bed volume) in 1 hr. After the column was washed with 150 ml of the equilibration buffer (20 mM Tris·HCl, pH 7.5/0.3 M NaCl), the retained protein (<5% total protein in retentate) was eluted with a modified linear gradient of increasing NaCl concentration. Fraction size was 3.8 ml, and flow rate during gradient elution was 108 ml/hr. Two microliters of the indicated fractions was transferred to microtiter wells and diluted to a final volume of 0.2 ml for assay of [³H]thymidine incorporation (incorp.) in BALB/MK cells.

Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala-Thr-Asn-Val. High background noise precluded an assignment for the first position. The initial yield was estimated to be 70 pmol, with a repetitive yield of 90%. A computer search using the FASTP program (24) revealed that the N-terminal amino acid sequence of KGF showed no significant homology to any protein in the National Biomedical Research Foundation data bank (May 1988, Release 16.0).

DISCUSSION

In the present study, we identified a human growth factor that has a distinctive specificity for epithelial cells. By using ultrafiltration, HSAC, and RP-HPLC or TSK molecular sieve chromatography, we isolated a quantity sufficient to permit detailed characterization of the physical and biological properties of this molecule. A single silver-stained band corresponding to a molecular mass of 28 kDa was detected in the active fractions from RP-HPLC, and the intensity of the band was proportional to the level of mitogenic activity in these fractions. A band indistinguishable from that obtained by RP-HPLC was seen in the active fractions from TSK chromatography. The purified protein stimulated DNA synthesis in epithelial cells at subnanomolar concentrations but failed to induce any thymidine incorporation in fibroblasts or endothelial cells at comparable or higher concentrations (up to 5 nM). Its unique target-cell specificity and N-terminal amino acid sequence lead us to conclude that it represents a previously unreported growth factor.

In a chemically defined medium, the purified factor was able to complement the insulin-like growth factor I/insulin growth requirement of BALB/MK cells and, therefore, must act through a signal-transduction pathway shared with EGF, TGF-α, and the FGFs. Moreover, the purified factor was more potent than any of the known epithelial cell mitogens in stimulating thymidine incorporation in BALB/MK cells. Preliminary evidence indicates that this factor is also capable of supporting proliferation of secondary human keratinocytes (our unpublished observations). In view of its preferential activity on the keratinocytes compared with other epithelial cells, we have provisionally named it KGF.

The ability of KGF to bind heparin may signify a fundamental property of this factor that has a bearing on its function *in vivo*. Growth factors with heparin-binding properties include aFGF (20–22), bFGF (19, 22), granulocyte–macrophage colony-stimulating factor (25), and interleukin 3 (25). Each of these is produced by stromal cells (25–27). Such factors appear to be deposited in the extracellular matrix or on proteoglycans coating the stromal cell surface (25, 28). It has been postulated that their storage, release, and contact with specific target cells are regulated by this interaction (25, 28). While mesenchymal-derived effectors of epithelial cell proliferation have also been described (29–31), their identities have not been elucidated. Its heparin-binding properties, release by human embryonic fibroblast stromal cells, and epithelial cell tropism provide KGF with all of the properties expected of such a paracrine mediator of normal epithelial cell growth. The partial amino acid sequence determined for this new growth factor should aid in efforts to molecularly clone its coding sequence and ascertain its relationship, if any, to known families of growth factors as well as its possible role in diseases characterized by epithelial cell dysplasia or neoplasia.

Note. Using oligonucleotide probes based on the N-terminal sequence reported in this manuscript, we have isolated clones encoding KGF from an M426 cDNA library. Sequence analysis reveals that KGF is a distinctive molecule with significant structural homology to the other five known members of the FGF family (32–35) (unpublished data).

We thank Janet Pumphrey for her excellent technical assistance in operating the gas-phase protein sequenator as well as Drs. John Sharefkin and Tim Fleming for providing and maintaining the human saphenous vein endothelial cells.

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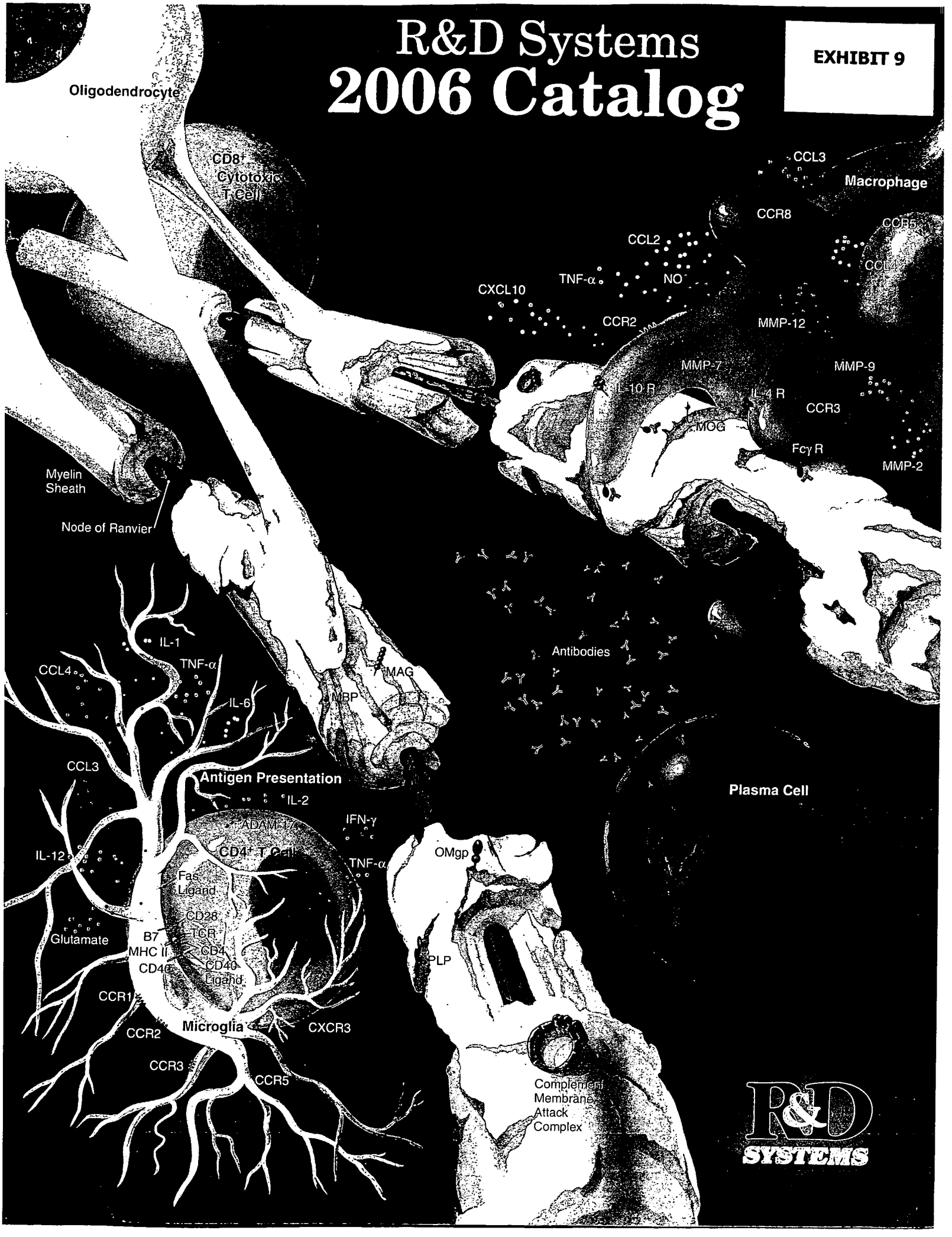

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EG-VEGF Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human EG-VEGF	None	Human EG-VEGF specific polyclonal goat IgG	• Western Blot	AF1209	100 µg	$355
Human EG-VEGF	Biotin	Human EG-VEGF specific polyclonal goat IgG	• Western Blot	BAF1209	50 µg	$380
Human EG-VEGF	None	Monoclonal mouse IgG_{2A}, clone # 188601	• Western Blot	MAB1209	500 µg	$295
Human/Mouse/Rat EG-VEGF	None	Monoclonal mouse IgG_{2B}, clone # 239727	• Western Blot	MAB2100	500 µg	$295
Rat EG-VEGF	None	Rat EG-VEGF specific polyclonal sheep IgG	• Western Blot	AF2100	100 µg	$355
Rat EG-VEGF	Biotin	Rat EG-VEGF specific polyclonal sheep IgG	• Western Blot	BAF2100	50 µg	$380

EGF (Epidermal Growth Factor)

EGF, also known as urogastrone, is the prototypic member of a family of growth factors derived from membrane-anchored precursors. All members of this family are characterized by the presence of at least one EGF structural unit in their extracellular domain. A wide variety of *in vitro* and *in vivo* biological effects have been ascribed to EGF and other members of the EGF family. EGF promotes proliferation and differentiation of mesenchymal and epithelial cells.

Activity is tested using the following assay:

1. Measured by its ability to stimulate ^{3}H-thymidine incorporation in a mouse fibroblast cell line Balb/3T3 (Rubin, J.S. *et al.*, 1991, Proc. Natl. Acad. Sci. USA **88**:415).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human EGF	Lyophilized without a carrier protein	*E. coli*-expressed	The ED_{50} is typically 0.1 - 0.4 ng/mL.	236-EG-200 236-EG-01M	200 µg 1 mg	$125 $405
Mouse EGF	Lyophilized without a carrier protein	*E. coli*-expressed	The ED_{50} is typically 0.04 - 0.16 ng/mL.	2028-EG-200	200 µg	$230

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human EGF	None	Human EGF specific polyclonal goat IgG	• Neutralization • Western Blot • Immunocytochemistry	AF236	100 µg	$355
Human EGF	Biotin	Human EGF specific polyclonal goat IgG	• ELISA Detection • Western Blot	BAF236	50 µg	$380
Human EGF	None	Monoclonal mouse IgG_1, clone # 10825	• Neutralization • Western Blot • Immunocytochemistry	MAB236	500 µg	$295
Human EGF	None	Monoclonal mouse IgG_1, clone # 10827	• ELISA Capture • Immunocytochemistry ○ Neutralization ○ Western Blot	MAB636	500 µg	$295
Mouse EGF	None	Mouse EGF specific polyclonal goat IgG	• Neutralization • Western Blot • Immunocytochemistry	AF2028	100 µg	$355
Mouse EGF	Biotin	Mouse EGF specific polyclonal goat IgG	• Western Blot	BAF2028	50 µg	$380
Mouse EGF	None	Monoclonal rat IgG_{2A}, clone # 262930	• Western Blot	MAB2028	500 µg	$295

ELISA & Activty Assays

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human EGF* *Quantikine Colorimetric Sandwich ELISA*	• Serum (S) • Platelet-poor Citrate, EDTA, and Heparin Plasma (P) • Cell Culture Supernate (C) • Urine (U)	10 µL (S) 200 µL (P, C) 10 µL (U)	< 0.7 pg/mL	3.9-250 pg/mL	DEG00 SEG00 PDEG00	1 Plate 6 Plates 50 Plates	$450 *Inquire* *Inquire*

**Contains Mercury, Dispose According to Local, State or Federal Laws.*

ELISA Development Kits

Analyte	Recommended Range	Catalog #	Reagents for*	Price
Human EGF	3.9-250 pg/mL	DY236	15 Plates	$645

**Also available in 45 plate Economy Packs for $1390.*

Fluorokine MAP Multiplex Kits for the Luminex Platform

Base Kit	Catalog #	Size	Price
Human Panel B	LUB000	1 Kit	$215

Analyte Kit	Sample Types	Catalog #	Size	Price
Human EGF	• Serum • EDTA and Heparin Plasma • Cell Culture Supernate	LUB236	100 Tests	$170

For more details on Multiplex products, please see page 875.

EGF R (Epidermal Growth Factor Receptor)

The epidermal growth factor receptor (EGF R) subfamily of receptor tyrosine kinases comprises four members: EGF R (also known as HER-1, ErbB1 or ErbB), ErbB2 (Neu, HER-2), ErbB3 (HER-3), and ErbB4 (HER-4). ErbB family members serve as receptors for the EGF family of growth factors.

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human EGF R *Extracellular Domain*	Lyophilized with a carrier protein	NS0-expressed	This protein's activity has not been tested.	1095-ER-002	2 µg	$99

FGF acidic (Fibroblast Growth Factor acidic)

FGF acidic, also known as FGF-1 and ECGF, is a member of the FGF family of mitogenic peptides. FGF acidic and basic, unlike the other members of the family, lack signal peptides and are apparently secreted by mechanisms other than the classical protein secretion pathway. The nucleotide sequence of human FGF acidic predicts a 155 amino acid protein.

Activity is tested using the following assay:

1. Measured by its ability to stimulate ^{3}H-thymidine incorporation in quiescent NR6R-3T3 fibroblasts (Rizzino, A. *et al.*, 1988, Cancer Res. **48**:4266; Thomas, K., 1987, Methods Enzymol. **147**:120).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Bovine FGF acidic	Lyophilized with a carrier protein	Bovine brain	The ED_{50} is typically 0.1 - 0.3 ng/mL.	132-FA-025	25 µg	$350
Human β-ECGF	Lyophilized with a carrier protein	*E. coli*-expressed	The ED_{50} is typically 0.1 - 0.3 ng/mL in the presence of 10 µg/mL heparin.	231-BC-025	25 µg	$230
Human FGF acidic	Lyophilized with a carrier protein	*E. coli*-expressed	The ED_{50} is typically 0.1 - 0.3 ng/mL in the presence of 10 µg/mL heparin.	232-FA-025	25 µg	$175

To order the carrier-free form, add "/CF" to the end of the catalog number (e.g., 132-FA-025/CF).

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Bovine FGF acidic	None	Polyclonal total rabbit IgG	• Neutralization • Western Blot	AB-32-NA	1 mg	$355
Human FGF acidic	None	Human FGF acidic specific polyclonal goat IgG	• Neutralization • Western Blot • Immunocytochemistry	AF232	100 µg	$355
Human FGF acidic	Biotin	FGF acidic specific polyclonal goat IgG	• Western Blot	BAF232	50 µg	$380
Human FGF acidic	None	Monoclonal mouse IgG_1, clone # 3117	• Western Blot	MAB232	500 µg	$295

ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human FGF acidic *Quantikine Colorimetric Sandwich ELISA*	• Serum • EDTA and Heparin Plasma • Cell Culture Supernate • Urine	50 µL	13.9 pg/mL	31.2-2000 pg/mL	DFA00B	1 Plate	$495

FGF-10 (Fibroblast Growth Factor 10)

FGF-10 is a member of the FGF family of heparin-binding mitogenic peptides. The human FGF-10 cDNA encodes a 208 amino acid residue protein with a hydrophobic amino-terminal signal peptide. Based on its *in vitro* biological activities and *in vivo* expression pattern, FGF-10 has been proposed to play unique roles in the brain, in lung development, wound healing and limb bud formation.

Activity is tested using the following assay:

1. Measured by its ability to stimulate the proliferation of a monkey epithelial cell line, 4MBr-5 (Rubin, J.S. *et al.*, 1989, Proc. Natl. Acad. Sci. USA **86**:802).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human FGF-10	Lyophilized with a carrier protein	*E. coli*-expressed	The ED_{50} is typically 100 - 300 ng/mL.	345-FG-025	25 µg	$250

To order the carrier-free form, add "/CF" to the end of the catalog number (e.g., 345-FG-025/CF).

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human FGF-10	None	Human FGF-10 specific polyclonal goat IgG	• Western Blot • Immunocytochemistry	AF345	100 µg	$355
Human FGF-10	Biotin	Human FGF-10 specific polyclonal goat IgG	• Western Blot	BAF345	50 µg	$380
Human FGF-10	None	Monoclonal mouse IgG_{2B}, clone # 186803	• Western Blot	MAB345	500 µg	$295

FGF-11 (Fibroblast Growth Factor 11)

FGF homologous factors exhibit sequence similarity with FGF family growth factors. FHFs are heparin-binding proteins like other FGFs, but they do not interact with FGF receptors. FHFs are intracellularly localized, and some have been shown to interact directly with cytoplasmic proteins. FGF-11, also known as FHF-3, is expressed in the pancreas and neuronal precursors.

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human FGF-11	None	Human FGF-11 specific polyclonal sheep IgG	• Western Blot	AF1634	100 µg	$355
Human FGF-11	Biotin	Human FGF-11 specific polyclonal sheep IgG	• Western Blot	BAF1634	50 µg	$380
Human FGF-11	None	Monoclonal mouse IgG_{2B}, clone # 224812	• Western Blot	MAB1634	500 µg	$295

FGF-12 (Fibroblast Growth Factor 12)

FGF-12 is a member of the FGF superfamily of heparin-binding mitogenic molecules characterized by the presence of a core, 120 amino acid (aa) β-trefoil structure. FGF-11, -12, -13, -14, originally termed FGF homologous factors (FHF) -3, -1, -2, and -4, respectively, form a subgroup within the FGF family. Human FGF-12/FHF-1 is synthesized as a 243 aa protein. It lacks a typical signal sequence and is considered cytoplasmic. It also possess an N-terminal bipartite nuclear localization signal at aa 11-18 and 28-38.

Activity is tested using the following assay:

1. Measured by its ability to bind immobilized rhFGF R4 or R5 in a functional ELISA.

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human FGF-12	Lyophilized with a carrier protein	*E. coli*-expressed	Binds with a linear range of 1.6 - 100 ng/mL.	2246-FG-025	25 µg	$315

To order the carrier-free form, add "/CF" to the end of the catalog number (e.g., 2246-FG-025/CF).

15(S)-HETE (15(S)-Hydroxyeicosatetraenoic Acid)

15(S)hydroxyeicosatetraenoic acid (15(S)-HETE) is the primary hydroxy derivative formed by the actions of 15-lipoxygenase and cyclooxygenase-1 on arachidonic acid. Increased levels of 15(S)-HETE are associated with asthma, rhinitis, chronic paranasal sinusitis and rheumatoid arthritis. 15(S)-HETE is thought to play a role in paracrine regulation of smooth muscle and lung neutrophil recruitment by incorporating into inositol phospholipids and modifying the regulation of intracellular calcium.

ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
15(S)-HETE *Colorimetric Competitive ELISA*	• Serum (S) • Citrate, EDTA, and Heparin Plasma (P) • Cell Culture Supernate (C) • Urine (U)	10 µL (S) 25 µL (P) 100 µL (C) 13 µL (U)	< 69 pg/mL	78.1-20,000 pg/mL	DE3000	1 Plate	$360

HGF (Hepatocyte Growth Factor)

Hepatocyte growth factor (HGF), also known as Hepatopoietin A, is mitogenic for a variety of cell types, including endothelial and epithelial cells, melanocytes, and keratinocytes. It is identical to scatter factor, a fibroblast-derived soluble factor that promotes the dissociation of epithelial and vascular endothelial cell colonies in monolayer cultures by stimulating cell migration. HGF is secreted as an inactive single chain precursor and is converted to the heterodimeric active form by HGF activator.

Activity is tested using the following assays:

1. Measured by its ability to stimulate ^{3}H-thymidine incorporation in an HGF-responsive epithelial cell line, 4MBr-5 (Rubin, J.S. *et al.*, 1991, Proc. Natl. Acad. Sci. USA **88**:415).
2. Measured by its ability to stimulate ^{3}H-thymidine incorporation in an mouse epithelial cell line, IMDC.

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human HGF	Lyophilized with a carrier protein	*Sf* 21-expressed	The ED_{50} is typically 20 - 40 ng/mL in assay 1.	294-HG-005 294-HG-025	5 µg 25 µg	$330 $1090
Human HGF	Lyophilized with a carrier protein	NS0-expressed	The ED_{50} is typically 20 - 40 ng/mL in assay 1.	294-HGN-005 294-HGN-025	5 µg 25 µg	$330 $1090
Mouse HGF	Lyophilized with a carrier protein	NS0-expressed	The ED_{50} is typically 10 - 30 ng/mL in assay 2.	2207-HG-025	25 µg	$315

To order the carrier-free form, add "/CF" to the end of the catalog number (e.g., 294-HG-025/CF).

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human HGF	None	Human HGF specific polyclonal goat IgG	• Neutralization • Western Blot • Immunocytochemistry	AF-294-NA	100 µg	$355
Human HGF	Biotin	Human HGF specific polyclonal goat IgG	• ELISA Detection • Western Blot	BAF294	100 µg	$380
Human HGF	None	Monoclonal mouse IgG_1, clone # 24612	• Neutralization • Western Blot	MAB294	500 µg	$295
Human HGF	None	Monoclonal mouse IgG_1, clone # 24516	• ELISA Capture • Western Blot	MAB694	500 µg	$295
Mouse HGF	None	Mouse HGF specific polyclonal goat IgG	• Western Blot • Immunocytochemistry	AF2207	100 µg	$355
Mouse HGF	Biotin	Mouse HGF specific polyclonal goat IgG	• Western Blot	BAF2207	50 µg	$380

HGF ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human HGF *Quantikine Colorimetric Sandwich ELISA*	• Serum • Citrate and EDTA Plasma • Cell Culture Supernate	50 µL	< 40 pg/mL	125-8000 pg/mL	DHG00 SHG00 PDHG00	1 Plate 6 Plates 50 Plates	$495 *Inquire* *Inquire*

ELISA Development Kits

Analyte	Recommended Range	Catalog #	Reagents for*	Price
Human HGF	125-8000 pg/mL	DY294	15 Plates	$645

Also available in 45 plate Economy Packs for $1390.

Fluorokine MAP Multiplex Kits for the Luminex Platform

Base Kit	Catalog #	Size	Price
Human Panel B	LUB000	1 Kit	$215

Analyte Kit	Sample Types	Catalog #	Size	Price
Human HGF	• Cell Culture Supernates • Serum • EDTA Plasma • Heparin Plasma	LUB294	100 Tests	$170

For more details on Multiplex products, please see page 875.

HGF Activator (HGFA)

HGF activator (HGFA) is a serine protease that cleaves single-chain HGF precursor, generating the active heterodimer. HGFA is secreted by the liver and normally circulates in the blood as an inactive zymogen, which can be activated by thrombin. The active protease is regulated by HGFA inhibitors.

Activity is tested using the following assay:

1. Cleavage of a fluorogenic peptide substrate, Mca-RPKPVE-NVal-WRK(Dnp)-NH_2 (Catalog # ES002).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human HGF Activator	Solution without a carrier protein	NS0-expressed	The specific activity is >15 pmole/min/µg.	1514-SE-010	10 µg	$315
Mouse HGF Activator	Solution without a carrier protein	NS0-expressed	The specific activity is >100 pmole/min/µg.	1200-SE-010	10 µg	$315

IFN-ω (Interferon omega)

Interferon-ω (IFN-ω), a type I interferon, is a monomeric glycoprotein distantly related in structure to IFN-α and IFN-β, but unrelated to IFN-γ. The human type I interferon receptor complex, which mediates the biological activity of IFN-α and IFN-β, also binds IFN-ω. The complex is composed of a 100 kDa ligand-binding subunit (IFN-α R1) and a 125 kDa subunit (IFN-α R2) that is involved both in ligand binding and signal transduction. IFN-ω is secreted by virus-infected leukocytes.

Activity is tested using the following assay:

1. Measured in the cytopathic effect inhibition assay (Rubinstein, S. *et al.*, 1981, J. Virol. **37**:755; Familletti, P.C. *et al.*, 1981, "A Convenient and Rapid Cytopathic Effect Inhibition Assay for Interferon" in *Methods in Enzymology*, S. Pestka, ed., Academic Press, New York, Vol. 78, 387-394).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human IFN-ω	Frozen with a carrier protein	*E. coli*-expressed	The specific activity is 1 x 10^8 units/mg.	11395-1	25 µg	$300

**Please call to inquire about carrier-free forms.*

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human IFN-ω	None	Monoclonal mouse IgG_1, clone # OMG-4	• ELISA Capture or Detection • Neutralization	21395-1	100 µg	$270

ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human IFN-ω *Colorimetric Sandwich ELISA*	• Serum • Plasma • Cell Culture Supernate	100 µL	NA	3-500 pg/mL	41395-1	1 Plate	$555

IGF-I (Insulin-like Growth Factor I)

IGF-I (also known as somatomedin C) and IGF-II (also known as somatomedin A and MSA) belongs to the family of insulin-like growth factors that are structurally homologous to proinsulin. IGF-I is a potent mitogenic growth factor that mediates the growth-promoting activities of growth hormone postnatally. In addition, recent evidence also suggests a role for IGF-I during embryonic growth and differentiation.

Activity is tested using the following assay:

1. Measured by its ability to induce proliferation of the human breast carcinoma cell line, MCF-7, under serum free conditions (Karey, K.P. *et al.*, 1988, Cancer Res. **48**:4083).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human IGF-I	Lyophilized without a carrier protein	*E. coli*-expressed	The ED_{50} is typically 1 - 3 ng/mL.	291-G1-050 291-G1-250	50 µg 250 µg	$170 $545
Mouse IGF-I	Lyophilized without a carrier protein	*E. coli*-expressed	The ED_{50} is typically 1 - 3 ng/mL.	791-MG-050	50 µg	$175

KGF/FGF-7 (Keratinocyte Growth Factor)

KGF (Keratinocyte growth factor), also known as FGF-7, is a member of the FGF superfamily of heparin-binding mitogenic molecules characterized by the presence of a core, 120 amino acid (aa) β-trefoil structure. Human KGF is a 194 amino acid (aa) precursor protein from which the N-terminal 31 aa are cleaved to generate the mature KGF.

Activity is tested using the following assay:

1. Measured by its ability to stimulate the proliferation of a monkey epithelial cell line, 4MBr-5 (Rubin, J.S. *et al.*, 1989, Proc. Natl. Acad. Sci. USA **86**:802).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Canine FGF-7/KGF	Lyophilized with a carrier protein	*E. coli*-expressed	The ED_{50} is typically 25 - 50 ng/mL.	1957-KG-025	25 µg	$315
Human FGF-7/KGF	Lyophilized with a carrier protein	*E. coli*-expressed	The ED_{50} is typically 15 - 25 ng/mL.	251-KG-010 251-KG-050	10 µg 50 µg	$285 $860

To order the carrier-free form, add "/CF" to the end of the catalog number (e.g., 132-FA-025/CF).

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human KGF	None	Human KGF specific polyclonal goat IgG	• Neutralization • Western Blot • Immunocytochemistry	AF-251-NA	250 µg	$315
Human KGF	Biotin	Human KGF specific polyclonal goat IgG	• ELISA Detection • Western Blot	BAF251	50 µg	$380
Human KGF	None	Monoclonal mouse IgG_1, clone # 29522	• Neutralization • ELISA Capture	MAB251	500 µg	$295
Human KGF	None	Monoclonal mouse IgG_1, clone # 29568	• Western Blot	MAB2511	500 µg	$295

ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human KGF/FGF-7 *Quantikine* *Colorimetric* *Sandwich ELISA*	• Serum • Citrate, EDTA and Heparin Plasma • Cell Culture Supernate	100 µL	< 15 pg/mL	31.2-2000 pg/mL	DKG00	1 Plate	$495

ELISA Development Kits

Analyte	Recommended Range	Catalog #	Reagents for*	Price
Human KGF/FGF-7	31.2-2000 pg/mL	DY251	15 Plates	$645

**Also available in 45 plate Economy Packs for $1390.*

KIAP *see Livin α*

TfR (Transferrin Receptor)

Transferrin Receptor (TfR) is the major mediator of iron uptake by cells. TfR is a transmembrane homodimer that binds and internalizes diferric transferrin. About 80% of total TfR is localized to erythroid progenitor cells. Soluble TfR (sTfR) arises from proteolysis of TfR leading to monomers that can be measured in plasma and serum. Levels of sTfR in plasma or serum have been used as an indirect measure of total TfR.

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human TfR *Extracellular Domain*	None	Human TfR specific polyclonal goat IgG	• Western Blot • Immunocytochemistry	AF2474	100 µg	$355
Human TfR	None	Monoclonal mouse IgG_1, clone # 29806	• Flow Cytometry	MAB2474	500 µg	$295
Human TfR	APC	Monoclonal mouse IgG_1, clone # 29806	• Flow Cytometry	FAB2474A	100 Tests	$305
Human TfR	PE	Monoclonal mouse IgG_1, clone # 29806	• Flow Cytometry	FAB2474P	100 Tests	$270

ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human sTfR *Quantikine IVD* Colorimetric Sandwich ELISA*	• Serum • Citrate, EDTA and Heparin Plasma	20 µL	< 0.5 nmol/L	0-80 nmol/L	DTFR1	1 Plate	$495

**For* In Vitro *Diagnostic Use. This product was manufactured in a plant whose quality management systems is certified to ISO 13485:1996/CMDCAS.*

TGF-α (Transforming Growth Factor alpha)

TGF-α is a member of the EGF family of cytokines. Membrane-bound pro-TGF-α is biologically active and seems to play a role in mediation of cell-cell adhesion and in juxtacrine stimulation of adjacent cells. Expression of TGF-α is widespread in tumors and transformed cells. TGF-α is also expressed in normal tissues during embryogenesis and in adult tissues, including pituitary, brain, keratinocytes and macrophages. Mature human TGF-α shows approximately 93% amino acid sequence identity with mouse or rat TGF-α and is not species-specific in its biological effects.

Activity is tested using the following assay:

1. Measured by its ability to stimulate ^{3}H-thymidine incorporation in a mouse fibroblast cell line, Balb/3T3 (Marquardt, H. *et al.*, 1984, Science **223**:1079).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human TGF-α	Lyophilized without a carrier protein	*E. coli*-expressed	The ED_{50} is typically 0.1 - 0.4 ng/mL.	239-A-100 239-A-500	100 µg 500 µg	$315 $990

Antibodies

Analyte	Label	Type	Applications • Recommended ○ Other	Catalog #	Size	Price
Human TGF-α	None	Human TGF-α specific polyclonal goat IgG	• Neutralization • ELISA Capture • Western Blot • Immunocytochemistry	AF-239-NA	100 µg	$355
Human TGF-α	Biotin	Human TGF-α specific polyclonal goat IgG	• ELISA Detection • Western Blot	BAF239	50 µg	$380

TGF-α ELISA & Activity Assay Kits

Analyte	Sample Types	Sample Requirement	Sensitivity	Range	Catalog #	Size	Price
Human TGF-α *Quantikine Colorimetric Sandwich ELISA*	• Serum • EDTA and Heparin Plasma • Cell Culture Supernate • Breast Milk	50 µL	< 7.10 pg/mL	15.6-1000 pg/mL	DTGA00	1 Plate	$495

ELISA Development Kits

Analyte	Recommended Range	Catalog #	Reagents for*	Price
Human TGF-α	7.8-500 pg/mL	DY239	15 Plates	$645

**Also available in 45 plate Economy Packs for $1390.*

TGF-β Family (Transforming Growth Factor beta)

TGF-β1 was originally identified as a secreted, disulfide-linked homodimeric protein that induced a transformed phenotype in mesenchymal cells. It has since been recognized that TGF-β1 is only one member of a superfamily of regulatory proteins consisting of a number of closely related proteins designated TGF-β2 - 5, as well as a number of distantly related proteins that include the inhibins and activins, Mullerian inhibitory substance, the BMPs and GDFs, and the GDNF family.

Activity is tested using the following assays:

1. Measured by its ability to inhibit mouse IL-4-dependent ^{3}H-thymidine incorporation by mouse HT-2 cells (Tsang, M. *et al.*, 1995, Cytokine **7**:389).
2. Measured by its ability to inhibit human TGF-β1-activity on mouse HT-2 cells (Tsang, M. *et al.*, 1995, Cytokine **7**:389).

Proteins

Protein	Form	Source	Activity	Catalog #	Size	Price
Human Latent TGF-β	Solution with a carrier protein	CHO-expressed	The ED_{50} is typically 20 - 60 ng/mL before acid activation and 0.15 - 0.5 ng/mL after acid activation in assay 1.	299-LT-005 299-LT-025	5 µg 25 µg	$285 $795
Human TGF-β1	Lyophilized with a carrier protein	Human platelets	The ED_{50} is typically 0.04 - 0.1 ng/mL in assay 1.	100-B-001 100-B-010	1 µg 10 µg	$285 $1725
Human TGF-β1	Lyophilized with a carrier protein	CHO-expressed	The ED_{50} is typically 0.04 - 0.1 ng/mL in assay 1.	240-B-002 240-B-010	2 µg 10 µg	$250 $805
Porcine TGF-β1	Lyophilized with a carrier protein	Porcine platelets	The ED_{50} is typically 0.1 - 0.2 ng/mL in assay 1.	101-B1-001 101-B1-010	1 µg 10 µg	$250 $1585
Human LAP (TGF-β1)	Lyophilized with a carrier protein	*Sf* 21-expressed	The ED_{50} is typically 20 - 40 ng/mL in assay 2.	246-LP-025	25 µg	$355
Human TGF-β1.2	Lyophilized with a carrier protein	*Sf* 21-expressed	The ED_{50} is typically 0.04 - 0.08 ng/mL in assay 1.	304-B3-001	1 µg	$375
Human TGF-β2	Lyophilized with a carrier protein	NS0-expressed	The ED_{50} is typically 0.1 - 0.3 ng/mL in assay 1	302-B2-002 302-B2-010	2 µg 10 µg	$350 $1140
Porcine TGF-β2	Lyophilized with a carrier protein	Porcine platelets	The ED_{50} is typically 0.1 - 0.3 ng/mL in assay 1.	102-B2-001	1 µg	$360
Human TGF-β3	Lyophilized with a carrier protein	*Sf* 21-expressed	The ED_{50} is typically 0.03 - 0.08 ng/mL in assay 1.	243-B3-002 243-B3-010	2 µg 10 µg	$325 $1055
Amphibian TGF-β5	Lyophilized with a carrier protein	*Sf* 21-expressed	The ED_{50} is typically 0.01 - 0.03 ng/mL in assay 1	245-B5-002 245-B5-010	2 µg 10 µg	$320 $850

To order the carrier-free form, add "/CF" to the end of the catalog number (e.g., 245-B5-010/CF).